

**THE ROLE OF PROSTANOIDS AND KININS IN
THE PHARMACOTHERAPY OF HEART FAILURE**

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References

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Declaration

I hereby declare that this thesis was composed by myself, the work contained was my own, I hold the degree of MB ChB from the University of Edinburgh, and I have not submitted this thesis in candidature for any other degree, diploma or professional qualification. The only exception to this is the work contained in chapter 4, which was conducted by a medical student, Mr Pardeep Jhund, under my direct guidance and supervision. Otherwise, all physiological measurements and blood sampling were conducted by myself, apart from the blood assays themselves. The blood assays in chapter 8 were kindly performed by Dr Ann Rumley and Professor Gordon Lowe, and the blood assays in chapter 9 were kindly performed by Dr Ian Young.

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Publications

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Davie AP, Dargie HJ, McMurray JJV. Role of bradykinin in the vasodilator effects of losartan and enalapril in patients with heart failure. *Circulation* 1999;**100**:268-273.

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Abstract

It has become clear that aspirin may have detrimental effects in heart failure. It has also become clear that angiotensin converting enzyme (ACE) inhibitors and, perhaps, angiotensin II type I receptor (AT1) antagonists may have beneficial "non-angiotensinergic" effects, not directly related to suppression of angiotensin II levels or blockade of the angiotensin II receptor. Some of these effects may be related to prostaglandins and/or kinins, such as bradykinin or substance P, and might therefore interact with the effects of aspirin. The studies described in this thesis aim to disentangle some of these interactions. The study in chapter 2 examined the effect of intra-arterial arachidonic acid on forearm blood flow in healthy volunteers and in patients with heart failure treated with an ACE inhibitor. We found that arachidonic acid was vasodilating and that this vasodilation was inhibited by 14 days oral aspirin, even a dose as small as 75mg daily. This does not prove that aspirin adversely affects vascular tone in these patients, but it does prove that it could. The study in chapter 3 examined the effect of aspirin on the immediate venodilator response to intravenous furosemide in patients with heart failure treated with an ACE inhibitor. We found that there was a small immediate venodilator response to intravenous furosemide, and we found that this vasodilator response was inhibited by 14 days oral aspirin, even a dose as small as 75mg daily. This proves that aspirin adversely affects the haemodynamic response to furosemide in these patients. The study in chapter 4 examined the effect of bradykinin and substance P on forearm blood flow in patients with heart failure treated with an ACE inhibitor, and the effect of aspirin. We found that bradykinin and substance P were potent vasodilators, but that neither was affected by aspirin. This implies that neither bradykinin nor substance P is

likely to contribute to a negative interaction between aspirin and ACE inhibitors. The study in chapter 5 examined the effects of bradykinin and its antagonist on forearm blood flow in patients with heart failure randomised to double-blind crossover treatment with ACE inhibitor or AT1 antagonist. Whilst we found that the response to bradykinin was enhanced by ACE inhibitor, as expected, we found no significant effect of bradykinin antagonism with an ACE inhibitor or an AT1 antagonist. These results point out a discrepancy between potentiation of exogenous bradykinin by ACE inhibitors and lack of potentiation of endogenous bradykinin by either ACE inhibitors or AT1 antagonists. This implies that bradykinin is unlikely to contribute to the non-angiotensinergic effects of ACE inhibitors (and unlikely to contribute to a negative interaction with aspirin). The study in chapter 6 examined the effect of intra-arterial bradykinin and angiotensin-(1-7) on forearm blood flow in patients with heart failure treated with an ACE inhibitor. We found that angiotensin-(1-7) had no significant effect on forearm blood flow, and no significant effect on the response to bradykinin. This implies that angiotensin-(1-7) is unlikely to contribute to the non-angiotensinergic effects of ACE inhibitors (and unlikely to contribute to a negative interaction with aspirin). The studies in chapter 7 and 8 examined the effect of ACE inhibitor and AT1 antagonist on endogenous fibrinolysis and nitrate/nitrite levels in patients with heart failure. We found no significant difference between the two treatments with regard to endogenous fibrinolysis or nitrate/nitrite levels after 5 weeks of enalapril 10mg bd or in the same 12 patients after 5 weeks of losartan 25mg bd. This means that the effect of ACE inhibitors on endogenous fibrinolysis and nitrate/nitrite levels is unlikely to reflect a non-angiotensinergic effect (and unlikely to interact with the effects of aspirin). In conclusion, we have shown that aspirin can

effect vascular tone in patients with heart failure, but that if it does interact with the effects of ACE inhibitors, it is unlikely to do so by an effect on bradykinin, substance P or angiotensin-(1-7). In addition, we have shown that there is a real discrepancy between potentiation of endogenous bradykinin and potentiation of exogenous bradykinin. Furthermore, we have been unable to find any difference between ACE inhibitors and AT1 antagonists in terms of endogenous fibrinolysis or nitrate/nitrite levels.

1.1 Nitric oxide and vascular tone

Vascular biology has come a long way from the time when the blood vessel was regarded as a passive conduit, to today, when the endothelium can be regarded as the largest endocrine organ in the body. The first major step on this pathway was the realisation of the fact that vasodilation to acetylcholine was endothelium-dependent, that is, literally dependent on the physical and vital integrity of vascular endothelium (Furchgott & Zawadzki, 1980). This rapidly led to the postulation of an endothelium-derived relaxing factor, which came to be known as EDRF, as the main mediator of such effects. It was some time before the final characterisation of EDRF as nitric oxide. This came with the demonstration that nitric oxide was indistinguishable from EDRF in terms of biological activity, stability, and susceptibility to both an inhibitor and a potentiator (Palmer *et al*, 1987). It is now clear that vascular tone is the end result of a complex interplay of simultaneously vasoconstricting and vasodilating factors. Some of these exert their effects on vascular smooth muscle directly (endothelium-independent) and some stimulate vascular endothelium to release other substances, which then influence vascular smooth muscle (endothelium-dependent) (Table 1.1). There are therefore a number of endothelium derived relaxing and contracting factors (Table 1.2), which mediate the effects of endothelium dependent vasodilators and vasoconstrictors. It is clear that a number of substances may have both endothelium-dependent and/or endothelium-independent effects, and both vasodilating and/or vasoconstricting effects in different conditions or under different circumstances, but endothelium derived relaxing factors are always vasodilating and endothelium derived contracting factors are always vasoconstricting (unless, like endothelin-1, it is also acting as an endothelium-dependent vasodilator). Furthermore,

impairment of endothelium-dependent (mainly vasodilator) responses and relative preservation of endothelium-independent responses has come to be recognised as the syndrome of endothelial dysfunction. Endothelial dysfunction is now well known as the hallmark of many pathophysiological states in cardiovascular disease, and indeed a number of other conditions as well (Table 1.3).

1.2 Prostacyclin and vascular tone

Over the past couple of decades much of the interest in vascular biology has focussed on what was first called EDRF and eventually characterised as nitric oxide. Over the same period, however, it has become clear that nitric oxide is far from being the only relaxing factor derived from the endothelium. Even when it transpired that EDRF and nitric oxide were one and the same thing, there remained evidence that prostanoids derived from arachidonic acid by cyclooxygenase, and an as yet uncharacterised substance specified as endothelium-derived hyperpolarising factor (EDHF), were also part of the communication between endothelium and associated smooth muscle. Indeed, it was a little before the sequence of events referred to above, that a substance first described as prostaglandin X (Moncada *et al*, 1976), and then as prostaglandin I₂ or prostacyclin (Whittaker *et al*, 1976), was shown to be a potent antiplatelet agent and a potent vasodilator (the name epoprostenol has also been proposed). When it was revealed that prostacyclin was released by endothelial cells (Weksler *et al*, 1977), the focus was initially on its antiplatelet effects (Moncada *et al*, 1977). Subsequently its vasodilator effects came to the fore (Dusting *et al*, 1977), especially in the pulmonary circulation (Kadowitz *et al*, 1978). It is now clear that prostacyclin fulfils the criteria of an endothelium-derived relaxing factor every bit

as much as nitric oxide.

1.3 Endothelium derived hyperpolarising factor

Somewhat later on than the above, the hyperpolarisation which had long been known to accompany vasodilation (Funaki & Bohr, 1967), was first of all perceived to be equally endothelium-dependent (Feletou & Vanhoutte, 1988), and then to be distinct from the effects of nitric oxide itself (Komori *et al*, 1988). Despite this early progress, endothelium-derived hyperpolarising factor (EDHF) has continued to elude definitive characterisation, and it is not even clear whether there is one EDHF or many. Early suggestions that EDHF might be ammonium ions (Feletou *et al*, 1989) or peroxide ions (Beny & von der Weid, 1991) have not been supported. Another line of enquiry suggests that EDHF might be derived from arachidonic acid via a cytochrome P450 dependent mechanism, and that its action is associated with calcium-activated potassium channel opening (Hecker *et al*, 1994). Two classes of substance have been postulated to fulfil these criteria, epoxyeicosatrienoic acids (EETs) (Campbell *et al*, 1996) and the endogenous cannabinoid anandamide (Randall *et al*, 1996). The data remains fragmentary and contradictory as far as these and other substances are concerned, however, and neither has been confirmed as EDHF (Zygmunt *et al*, 1996, Plane *et al*, 1997). More recently, it has been suggested that EDHF might be C-type natriuretic factor but this does not seem to be the case either (Barton *et al*, 1998). The most recent line of enquiry suggests that EDHF may be a particle even simpler than the simple molecule of nitric oxide, i.e., the potassium ion (Edwards *et al*, 1998). Whether or not EDHF is finally pinned down to a single substance, it seems likely that the number of recognised endothelium derived relaxing

factors will continue to proliferate in the years ahead, as it has to date.

1.4 Thromboxane and vascular tone

Blood vessels are obviously not just under the influence of agonists that cause vasodilation, but also agonists that cause vasoconstriction. This is reflected in the fact that there are not just endothelium derived relaxing factors but also endothelium derived contracting factors. The principal EDCF has been characterised as endothelin-1 (Yanagisawa *et al*, 1988) and this has been a focus of almost as much interest as nitric oxide, having been discovered around the same time. However, just as it has become clear that there is a family of endothelium derived relaxing factors, it has also become clear that there is a family of endothelium derived contracting factors. Again, metabolites of arachidonic acid are notable players in this aspect of endothelial control. Indeed, just as arachidonic acid may be metabolised by endothelial cells to produce the EDRF prostacyclin (Weksler *et al*, 1977), it may alternatively be metabolised by endothelial cells to produce the EDCF thromboxane (Ingberman-Wojenski *et al*, 1981). This makes the role of arachidonic acid and its metabolites in vascular biology in general and in endothelial control of smooth muscle in particular all the more interesting.

1.5 Neurohormonal activation and endothelial dysfunction

We are particularly interested in the syndrome of congestive heart failure due to left ventricular systolic dysfunction. It has long been recognised that heart failure is characterised by an abnormality of vascular tone with excessive vasoconstriction (Zelis *et al*, 1974). At one level this is simply due to the activation of multiple

endocrine pathways that has come to be known as neurohormonal activation (Thomas *et al*, 1978). Initially the vasoconstriction and fluid retention that this produces are compensatory mechanisms designed to maintain blood pressure and renal perfusion. In both the short and long term, however, fluid retention and vasoconstriction are an inappropriate response to the development of heart failure. Despite some initial controversy, it has come to be accepted that this process contributes to progression of the disease and is a reasonable target for therapy aimed at interruption of this progression (Swedberg, 1988; Cohn, 1988; Packer, 1992). At the same time, endothelial dysfunction has also come to be a well-recognised facet of the syndrome of heart failure (Kaiser *et al*, 1989). It is obvious from Table 1.3 that many of the individual causes of endothelial dysfunction (hypertension, diabetes, hyperlipidaemia, smoking, ageing and atherosclerosis) are part of the syndrome of heart failure (it is even hypothesised that endotoxaemia may also be an unexpected facet of the syndrome of heart failure, Niebauer *et al*, 1999). Indeed, many of these factors contribute to the development of heart failure itself for the majority of patients with heart failure due to ischaemic heart disease. It is thus difficult to separate the effects of one cause of endothelial dysfunction from the effects of another, especially in patients with heart failure. It is also immediately obvious that there must be a close communication between endothelial dysfunction and neurohormonal activation, to the extent that it must be difficult to disentangle the effects of one from the other, or to know to what extent one causes the other.

1.6 Other metabolites of arachidonic acid

The question also arises, to what extent abnormalities of prostaglandin

metabolism might arise from, or contribute towards, endothelial dysfunction. As we have already seen, prostanoids participate in endothelial control of smooth muscle relaxation (in the form of the endothelium derived relaxing factor, prostacyclin) and contraction (in the form of the endothelium derived contracting factor, thromboxane). We have also seen (Table 1.1) that the common precursor of all prostanoids, arachidonic acid, has been described as an endothelium-dependent vasodilator (De Mey & Vanhoutte, 1982), an endothelium-dependent vasoconstrictor (De Mey & Vanhoutte, 1982), an endothelium-independent vasodilator (Pinto *et al*, 1986), or an endothelium-independent vasoconstrictor (Boulanger & Vanhoutte, 1993). This unique versatility (see Table 1.1 for comparisons) is a consequence of the fact that arachidonic acid is not itself an agonist at any known receptor, but a common precursor of the substances which are. Arachidonic acid is released by the action of phospholipase on phospholipid from the cell membrane and metabolised to a number of different sorts of receptor agonist, many of them vasoactive in their own right (Figure 1.1). Apart from prostacyclin and thromboxane, there is prostaglandin H₂ (Toda *et al*, 1988), prostaglandin F_{2α} (Ducharme *et al*, 1968), leukotriene C₄ and D₄ (Sakuma *et al*, 1987), 15-hydroxyeicosatetraenoic acid (15-HETE) and 15-hydroperoxyeicosatetraenoic acid (15-HPETE) (Van Diest *et al*, 1986), and 20-hydroxyeicosatetraenoic acid (20-HETE) (Escalante *et al*, 1989). This is all quite apart from the potential role of EETs and/or anandamide as alluded to earlier.

1.7 Heart failure and prostaglandins

It follows from the above that there are many different ways in which abnormalities of prostaglandin metabolism might contribute to the endothelial

dysfunction (or indeed the smooth muscle cell dysfunction ?) of heart failure. This rather begs the question, however, of whether or not there is any evidence that they do so. There is evidence that interfering with prostaglandins in heart failure is detrimental (Walshe & Venuto, 1979), and that exogenous prostaglandin administration may be therapeutic in heart failure (Awan *et al*, 1981). Despite this, the most well known attempt to demonstrate a mortality benefit (with long term intravenous administration of synthetic prostacyclin) actually demonstrated excess mortality (Califf *et al*, 1997). There appears to be rather less evidence that disorders of prostaglandin production and function are an inherent part of the syndrome of heart failure. Dzau *et al* (1984) found that heart failure was associated with increased plasma levels of prostaglandin E₂-metabolite and 6-keto-prostaglandin F_{1α} (a chemically stable metabolite of prostacyclin), and that the association was especially strong in patients with hyponatraemia and activation of the renin-angiotensin-system (in whom administration of the cyclooxygenase inhibitor indomethacin was associated with haemodynamic deterioration). The findings of this study were corroborated by a later study (Punzengruber *et al*, 1986) which found very similar associations with plasma levels of bicyclo-prostaglandin E₂ metabolite (a more stable prostaglandin E₂ degradation product). One animal study (Holmer *et al*, 1987) found that plasma levels of 6-keto-prostaglandin F_{1α} increased in 4 out of 6 dogs during the first 4 days of pacing-induced heart failure. An exercise study (Ueyuma *et al*, 1993) found not only that plasma levels of 6-keto-prostaglandin F_{1α} were elevated, but that their exercise-induced elevation was further increased in patients with heart failure. The most recent study (Castellani *et al*, 1997) found a correlation between functional class of heart failure and urinary daily excretion of thromboxane and prostaglandin F_{2α}.

There therefore does seem to be evidence of an association between heart failure, especially more severe heart failure (made manifest by hyponatraemia, renin-angiotensin-system activation and/or worse functional class), and elevation of levels of both vasodilator and vasoconstrictor prostaglandins. Since both vasodilator and vasoconstrictor prostaglandins, as we have seen, may be released by endothelium, the question certainly does arise to what extent the increased release of such substances might represent an unimpaired or even an enhanced endothelial response. Such a mechanism could be a compensation for other impaired aspects of endothelial function, or an effector mechanism of neurohormonal activation (or both simultaneously). This aspect of vascular function in heart failure is clearly deserving of further study.

1.8 Vascular effects of aspirin

In connection with the above, we are particularly interested in the vascular effects of aspirin in patients with heart failure. Aspirin (acetylsalicylic acid) is an inhibitor of prostaglandin synthase (cyclooxygenase) (Vane, 1971) which is widely used in cardiovascular pharmacology for its antiplatelet effects. This means that, deliberately or not, it is used in many patients with heart failure as a consequence of ischaemic heart disease. Until recently, it has always been assumed that aspirin has no direct vascular effects, in heart failure, or in any other condition. However, it follows from the preceding considerations that aspirin may have very important vascular effects in heart failure. Only very recently has the idea that the effects of aspirin in heart failure may not be entirely beneficial received an airing (Cleland *et al*, 1995). As it turns out, however, aspirin does not appear to have any direct immediate vascular

effects on its own. The reason for this is not clear, but it has necessitated the examination of more complex interactions to pin down the role of aspirin therapy in heart failure.

1.9 Vascular effects of ACE inhibitors

We are also particularly interested in the vascular effects of inhibitors of angiotensin converting enzyme (ACE). Over recent years, ACE inhibitors have consolidated their position as a cornerstone of the evidence-based treatment of heart failure (Garg & Yusuf, 1995). More recently, the idea that ACE inhibitors might owe some of their beneficial effects to restoration of impaired endothelial function has gained some currency (Nakamura *et al*, 1991, Mancini *et al*, 1996). Despite this, it is not at all clear whether such an effect would be due to suppression of angiotensin II levels or to potentiation of bradykinin levels or both (ACE is responsible for the breakdown of bradykinin as well as the production of angiotensin II). It is certainly possible that not all of the beneficial effects of ACE inhibitors are necessarily due to suppression of angiotensin II levels. Indeed, ACE inhibitors may not even necessarily be very good at suppressing angiotensin II levels, because of non-ACE pathways of angiotensin II generation (Van den Meiracker *et al*, 1992; Padmanabhan *et al*, 1999). Some of the beneficial effects of ACE inhibitors may therefore be "non-angiotensinergic", or at least "non-angiotensin-II-ergic".

1.10 Differences between ACE inhibitors and AT1 antagonists

More recently, angiotensin II type I receptor (AT1) antagonists have become available. Whilst initially perceived as being very similar to ACE inhibitors, with time

it has become clearer that they may be very different. The reason for this is the extraordinary complexity of the renin-angiotensin-system. It has become clear that angiotensin I and II are not the only angiotensins. In fact, as shown in Table 1.4, at least sixteen different angiotensins may result from the action of ACE, or other endopeptidases, or carboxypeptidase, or aminopeptidase, on angiotensin I. Most of these have been detected in human plasma (Semple *et al*, 1976; Lawrence *et al*, 1990), and many of them have been demonstrated to be vasoactive, albeit in animals. It is also clear that the level of many of these substances may be markedly increased by ACE inhibitors (Lawrence *et al*, 1990). The effect of AT1 antagonists on the levels of such substances is completely unknown. It has also become clear that the AT1 receptor and the AT2 receptor are not the only angiotensin receptors. In fact, as shown in Table 1.5, there are probably at least five different subtypes of angiotensin receptor, and some of the less well known ones probably account for the action of some of the less well known angiotensins. Given this complexity, it is not surprising that it is not clear whether AT1 antagonists are better than (e.g., Pitt *et al*, 1997), just the same as (e.g., Pitt *et al*, 1999), or not as good as (e.g., McKelvie *et al*, 1999) ACE inhibitors.

1.11 Interaction between aspirin and ACE inhibitors

If there are significant differences between ACE inhibitors and AT1 antagonists, they are likely to result from effects on bradykinin, or non-angiotensin II angiotensins, or non-AT1 angiotensin receptors. The effects of bradykinin are frequently said to be prostaglandin dependent - it is not known whether any of these other effects are or not. It follows from this that any differences between ACE

inhibitors and AT1 antagonists stand a good chance of being prostaglandin-dependent. At present it is not clear whether ACE inhibitors and AT1 antagonists do have effects distinct from one another or not, but if they do have, they might then be expected to interact differently with the effects of aspirin. There have been a number of small-scale haemodynamic studies to suggest an interaction between aspirin and ACE inhibitors. Starting with Hall *et al* in 1992, the results of these have been somewhat contradictory and are discussed in chapter 3 (section 3.4.7). There have also been a number of post-hoc analyses of the interaction of aspirin and ACE inhibitors in large-scale randomised controlled trials (of ACE inhibitors or other agents). Results from SOLVD have been interpreted by some as showing an interaction (Cleland *et al*, 1995; 1996), but by others as showing no interaction (Pitt, 1993; Al-Khadra *et al*, 1998). Results from CONSENSUS-2 have been interpreted as showing an interaction (Nguyen *et al*, 1997), but CONSENSUS-2 was negative overall, and therefore very much at odds with other studies of ACE inhibitors. Results from CATS have been interpreted by some as showing an interaction (Oorsterga *et al*, 1998), but by others as showing no interaction (Davie, 1999). Results from BIP have been interpreted as showing no interaction (Leor *et al*, 1999), but enthusiasm for these results must be tempered by the fact that BIP was a trial of lipid-lowering therapy in patients with coronary disease but without heart failure. Unfortunately it remains highly unlikely that there will ever be a large-scale randomised controlled trial (2 x 2 factorial design) of the interaction of aspirin and ACE inhibitors or angiotensin antagonists in patients with heart failure.

1.12 Interaction between aspirin and AT1 antagonists

There is currently no evidence at all on whether or not there is an interaction between aspirin and AT1 antagonists. Work in this field might be furthered, by direct examination of interactions between aspirin and ACE inhibitors or between aspirin and AT1 antagonists, or by closer examination of the potential differences between ACE inhibitors and AT1 antagonists.

1.13 Aims and objectives

It is with the above considerations in mind that we have been interested in examining the vascular effects of aspirin and ACE inhibitors and AT1 antagonists, and the interactions between them, in patients with heart failure. We have chosen to use the technique of venous occlusion plethysmography for measurement of forearm blood flow in patients with heart failure. This has the strength of examining clinical pharmacology in humans rather than in animals, *in vivo* rather than *in vitro*, in real patients rather than in some model of heart failure, and in a single predictable vascular bed, rather than the more complicated and more unpredictable whole body. The problem with this is that despite some isolated examples to the contrary (Bank *et al*, 1991, Duffy *et al*, 1998), the consensus of opinion is that aspirin does not have any direct immediate effects on its own in this situation, and nor do ACE inhibitors (Benjamin *et al*, 1989). The reasons for this are not immediately obvious, but might be something to do with a dissociation between immediate and delayed effects. Whatever the reasons, it clearly means that we need to examine rather more complex interactions to shed light on the subject.

1.14 Current studies

The current series of experiments was designed to attempt to delineate more clearly the role of arachidonic acid and its metabolites in the vasculature of patients with heart failure, and their modulation by aspirin therapy and/or ACE inhibitor therapy and/or AT1 antagonist therapy. In chapter 3, we examine the effects of arachidonic acid itself in normals, in patients with heart failure, and the effect of aspirin on the response to arachidonic acid in patients with heart failure (all treated with ACE inhibitor). In chapter 4, we examine the effects of the same doses of aspirin on the venodilator response to furosemide in patients with heart failure (seeking clinical validation of the effect observed with arachidonic acid). In chapter 5, we examine the effects of bradykinin and substance P (bradykinin is the principal candidate for non-angiotensinergic effects of ACE inhibitors and may or may not have prostaglandin-dependent effects itself - the same is possibly true of substance P) in patients with heart failure, and the effects of aspirin on this response. In chapter 6, we contrast the effects of bradykinin with the effects of bradykinin antagonism in patients with heart failure, and the effects of ACE inhibition and AT1 antagonism on this response. In chapter 7, we examine the effects of angiotensin-(1-7) (angiotensin-(1-7) is the principal candidate for non-angiotensin-II-ergic angiotensinergic effects of ACE inhibitors and may or may not have prostaglandin-dependent effects itself) in patients with heart failure. In chapter 8, we examine the effects of ACE inhibition and AT1 antagonism on plasma fibrinolytic activity in patients with heart failure. Plasma fibrinolytic activity is a further dimension of endothelial function, which is a potentially important substrate for the effects of aspirin as well as ACE inhibitors and/or AT1 antagonists. In chapter 9, we examine the effects of ACE inhibition and

AT1 antagonism on plasma nitrate concentrations, which appear to represent an overall marker of the level of endothelial function, in patients with heart failure.

Table 1.1 Endothelium dependent & independent vasodilators & vasoconstrictors

Endothelium dependent vasodilators

Acetylcholine (Furchgott & Zawadzki, 1980)
 PDGF (Coughlin *et al*, 1980)
 ADP/ATP (De Mey & Vanhoutte, 1981)
 Thrombin (De Mey & Vanhoutte, 1981)
 Bradykinin (Cherry *et al*, 1982)
Arachidonic Acid (De Mey & Vanhoutte, 1982)
 Substance P (Angus *et al*, 1983)
 Serotonin (Cocks & Angus, 1983)
 α -agonists (Cocks & Angus, 1983)
 Histamine (Van de Voorde & Lausen, 1983)
 Vasopressin (Katusic *et al*, 1984)
 Angiotensin II (Toda, 1984)
 Oxytocin (Katusic *et al*, 1986)
 β agonists (Carvalho *et al*, 1987)
 Vasoactive Intestinal Peptide (Thom *et al*, 1987)
 Endothelin (Cocks *et al*, 1989)
 Platelet Activating Factor (Chiba *et al*, 1990)
 CGRP (Samuelson & Jernbeck, 1991)
 Adrenomedullin (Hirata *et al*, 1995)

Endothelium dependent vasoconstrictors

Thrombin (De Mey & Vanhoutte, 1982)
Arachidonic Acid (De Mey & Vanhoutte, 1982)
 Serotonin (Lamping *et al*, 1985)
 Acetylcholine (Luscher & Vanhoutte, 1986)
 α -agonists (Usui *et al*, 1987)
 Neuropeptide Y (Daly & Hieble, 1987)
 ADP/ATP (Shirahase *et al*, 1988)
 Angiotensin II (Manabe *et al*, 1989)
 Histamine (Rosenblum *et al*, 1990)
 Somatostatin (Shirahase *et al*, 1993)
 Platelet Activating Factor (Gao *et al*, 1995)
 Substance P (Shirahase *et al*, 1995)
 Endothelin (Taddei & Vanhoutte, 1993)
 Urotensin-II (Ames *et al*, 1999)

PDGF = platelet-derived growth factor; ADP = adenosine diphosphate; ATP = adenosine triphosphate; CGRP = calcitonin gene-related peptide; AMP = adenosine monophosphate; PACAP = pituitary adenylate cyclase-activating polypeptide

Endothelium independent vasodilators

Atrial Natriuretic Factor (Winqvist *et al*, 1984)
Arachidonic Acid (Pinto *et al*, 1986)
 Vasoactive Intestinal Peptide (Sata *et al*, 1986)
 β agonists (Carvalho *et al*, 1987)
 Adenosine/AMP (Shirahase *et al*, 1988)
 PACAP (Warren *et al*, 1991)
 CGRP (Samuelson & Jernbeck, 1991)
 Adrenomedullin (Hirata *et al*, 1995)

Endothelium independent vasoconstrictors

Thrombin (Ku, 1982)
 Serotonin (Cocks & Angus, 1983)
 α -agonists (Cocks & Angus, 1983)
 Acetylcholine (Griffith *et al*, 1984)
 Histamine (Griffith *et al*, 1984)
 Angiotensin II (Lamping *et al*, 1985)
 Vasopressin (Katusic *et al*, 1986)
 Oxytocin (Katusic *et al*, 1986)
 Neuropeptide Y (Pernow & Lundberg, 1988)
 Substance P (Beny & Brunet, 1988)
Arachidonic Acid (Boulanger & Vanhoutte, 1993)

Table 1.2 Endothelium derived relaxing factors & contracting factors

Endothelium derived relaxing factors

Nitric oxide (Palmer *et al*, 1987)

Prostacyclin (Weksler *et al*, 1977)

"Endothelium Derived Hyperpolarising Factor" (Komori *et al*, 1988)

<i>FOR</i>	<i>AGAINST</i>
ammonium ions ?	not (Feletou <i>et al</i> , 1989)
hydrogen peroxide ?	not (Beny & von der Weid, 1991)
EETs ? (Campbell <i>et al</i> , 1996)	or not ? (Zygmunt <i>et al</i> , 1996)
Anandamide ? (Randall <i>et al</i> , 1996)	or not ? (Plane <i>et al</i> , 1997)
C-type natriuretic peptide ?	probably not (Barton <i>et al</i> , 1998)
K ⁺ ions ? (Edwards <i>et al</i> , 1998)	

Endothelium derived contracting factors

Endothelin-1 (Yanagisawa *et al*, 1988)

Thromboxane (Ingerman-Wojenski *et al*, 1981)

Table 1.3 **Conditions associated with endothelial dysfunction**

Hypertension (Winqvist *et al*, 1984)

Diabetes mellitus (Oyama *et al*, 1986)

Hyperlipidaemia (Verbeuren *et al*, 1986)

Atherosclerosis (Ludmer *et al*, 1986)

Ageing (Soltis, 1987)

Heart failure (Kaiser *et al*, 1989)

Endotoxaemia (Siegfried *et al*, 1992)

Smoking (Celermajer *et al*, 1993)

Cyclosporin therapy (Gallego *et al*, 1993)

Hyperhomocystinaemia (Celermajer *et al*, 1993)

Renal failure (Nakayama *et al*, 1994)

Liver cirrhosis (Ferro *et al*, 1996)

Table 1.4 The family of angiotensins

Angiotensin I = Angiotensin-(1-10) = Asp Arg Val Tyr Ile His Pro Phe His Leu

Angiotensin-(1-9) = Asp Arg Val Tyr Ile His Pro Phe His

Angiotensin II = Angiotensin-(1-8) = Asp Arg Val Tyr Ile His Pro Phe

Angiotensin-(1-7) = Asp Arg Val Tyr Ile His Pro

Angiotensin-(1-6) = Asp Arg Val Tyr Ile His

Angiotensin-(1-5) = Asp Arg Val Tyr Ile

Angiotensin-(1-4) = Asp Arg Val Tyr

Angiotensin-(2-10) = Arg Val Tyr Ile His Pro Phe His Leu

Angiotensin-(2-9) = Arg Val Tyr Ile His Pro Phe His

Angiotensin III = Angiotensin-(2-8) = Arg Val Tyr Ile His Pro Phe

Angiotensin-(2-7) = Arg Val Tyr Ile His Pro

Angiotensin-(2-6) = Arg Val Tyr Ile His

Angiotensin-(2-5) = Arg Val Tyr Ile

Angiotensin IV = Angiotensin-(3-8) = Val Tyr Ile His Pro Phe

Angiotensin-(3-7) = Val Tyr Ile His Pro

Angiotensin-(4-8) = Tyr Ile His Pro Phe

Angiotensin-(5-8) = Ile His Pro Phe

Bradykinin (for comparison) = Arg Pro Pro Gly Phe Ser Pro Phe Arg

Substance P (for comparison) = Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met

Table 1.5 The family of angiotensin receptors (& *their preferred ligands*)

Angiotensin II type 1 (AT1) receptor	<i>(Angiotensins II & III)</i>
AT1A receptor	
AT1B receptor	
AT1C receptor ? = AT3 receptor ?	
Angiotensin II type 2 (AT2) receptor	<i>(Angiotensins II & III)</i>
Angiotensin II type 3 (AT3) receptor ? = AT1C receptor ?	
Angiotensin II type 4 (AT4) receptor	<i>(Angiotensins III, IV & 1-7)</i>
Angiotensin-(1-7) receptor	<i>(Angiotensin 1-7)</i>

2. Methods

2.1 Introduction

We chose to use the technique of venous occlusion plethysmography for measurement of forearm blood flow (and forearm venous capacitance) in patients with heart failure. We were therefore examining clinical pharmacology in humans rather than in animals. Findings in humans are inherently more important than findings in animals as it is unpredictable to what extent responses may differ between the two, and generally it is humans we are interested in *treating*, more than animals. We were also examining clinical pharmacology *in vivo* rather than *in vitro*. Findings *in vivo* are inherently more important than findings *in vitro*, as it is unpredictable to what extent specimen preparation may alter "normal" physiology and pathophysiology (just as Furchgott & Zawadzki discovered when they realised that vasodilation to acetylcholine was endothelium-dependent). We were also examining clinical pharmacology in patients with heart failure rather than some model of heart failure. Findings in patients with heart failure are more important than findings in models of heart failure, as, despite the prevalence of heart failure, efforts have yet to yield a really convincing model of heart failure which replicates it in all its complexity. We were also examining clinical pharmacology in a single vascular bed rather than at the whole body level. Whilst testing potentially untried substances it is obviously safer to do so with small doses at the local level rather than with larger doses at the systemic level. Apart from this, there is the fact that different vascular beds may very well differ in their response to any given substance (presumably due to phenotypic differences in gene expression) and may cancel each other out, or at least make systemic effects very much more difficult to interpret. Although systemic studies are therefore important, local studies (preferably in more than one vascular bed) are just

as important, and probably a necessary preliminary to the former (especially when testing potentially untried substances).

2.2 Background

Plethysmography is the method of measuring variations in the size of parts of the body (from Greek, *plethynein* to increase), and venous occlusion plethysmography is the method of measuring arterial inflow by measuring the increases in size of parts of the body which result from occlusion of venous outflow. It will be immediately obvious that the method is most easily applied to appendages such as limbs or digits, and indeed, the method is most often used for measurement of blood flow in the calf, the thigh, or as in this case, the forearm. When first developed, the technique required the limb in question to be encased in a box containing air or water, so that the displacement of air or water, or changes in pressure, could be measured to give the change in volume of the limb in question (Whitney *et al*, 1953). This technique was obviously extremely cumbersome and subject to interference from multiple sources, and hence difficult for the investigator to manage and equally difficult for the subject to endure. The development of a strain gauge which could measure changes in size more directly and more easily transformed the technique and made it much more widely applicable (Hokanson *et al*, 1975). Originally this took the form of a hollow loop of elastic filled with liquid mercury, the electrical conductance of which depended on its degree of distension (a variety of piezoelectric effect). This is simply looped around the limb in question and a voltage plethysmograph measures the minute changes in resistance which can be calibrated to equate with changes in limb size. Over subsequent years the strain gauge has evolved into a hollow loop of

Silastic (for durability) filled with a liquid alloy of indium and gallium (for safety), but the principle is exactly the same.

2.3 Ward set-up

For measurement of forearm blood flow by venous occlusion plethysmography the subject attends having abstained from alcohol, caffeine and tobacco for 24 hours, and from food for 6 hours, and having emptied the bladder. It is obviously important that the subject has abstained from any potentially vasoactive stimulants, but it is also important that digestion is not active, as this could divert blood from the limbs to the gastro-intestinal tract. For this reason, all our studies were conducted in the early afternoon, with subjects having breakfasted, but having abstained from lunch. Studies can be quite time-consuming, and it is important that the patient is as comfortable as possible. Nothing upsets delicate haemodynamic measurements like an uncomfortably full bladder (quite apart from the fact that it may lead to early termination of the study, and a loss of co-operation for future studies). The subject is accommodated in a quiet research ward maintained at a constant temperature of 23-25°C. It is important that the ward is quiet and that its peace is not disturbed, as any sudden interruptions can have surprisingly significant effects on blood flow. It is also important that the ward is a comfortable temperature and that that temperature is maintained, as any variations in temperature will affect blood flow (generally increases in temperature cause vasodilation and decreases in temperature cause vasoconstriction). The subject or patient lies on a comfortable bed for at least 20 minutes before any drug infusion while haemodynamics adjust to the supine position. It is important that the subject or patient is as comfortable as possible, as studies can

be quite time-consuming, and it is obviously important that the results of the study are not confounded by unstable baseline haemodynamics.

2.4 Subject set-up

The subject's forearms are both supported on pillows, cushions or foam pads so that they are at or above the level of the right atrium. This assists venous outflow by means of gravity, so that after the cessation of venous occlusion the forearm quickly returns to normal, prior to the next measurement. Pressure cuffs are applied to both upper arms (Hokanson SC10, PMS Instruments, Maidenhead, Berkshire) for venous occlusion ($\sim 40\text{mmHg}$), and to both wrists (Hokanson SC5, PMS Instruments) for arterial occlusion ($\sim 220\text{mmHg}$) and isolation of the hand circulation. The reason for this is that whilst the forearm circulation is mostly muscle perfusion, the hand circulation is mostly skin perfusion, and behaves differently, and indeed, somewhat unpredictably. The pressure cuffs are connected to a pair of rapid cuff inflators (Hokanson E20, PMS Instruments) supplied by a commercially available air source (Hokanson AG101, PMS Instruments). Indium/gallium-in-Silastic strain gauges (Hokanson forearm set, PMS Instruments) of an appropriate size are applied to the widest aspect of each forearm and connected to a voltage plethysmograph (Hokanson plethysmograph, PMS Instruments).

2.5 Intrabrachial cannulation

After local anaesthesia with 1% lidocaine (Phoenix Pharma, Gloucester), a 27-gauge steel needle (Terumo Medical Corporation, Tokyo, Japan) is inserted into the brachial artery (non-dominant, to minimise the consequences in case of the very slight

risk of complications), and connected to a constant rate infusion pump (IVAC P1000, Alaris Medical Systems, San Diego, California, USA) via a 16-gauge epidural catheter (Portex Limited, Hythe, Kent). Physiological saline solution (0.9%, Baxter Healthcare Corporation, USA) is infused at 1 ml/min for at least 20 minutes before any drug infusion. Forearm blood flow often increases and occasionally decreases in the infused arm after insertion of the intrabrachial needle, and it is obviously important that blood flow equilibrates first, so that changes in baseline blood flow do not confound the results of the study.

2.6 Measurement of blood flow

To measure blood flow, the wrist cuffs are inflated to supra-systolic pressure (~ 220 mmHg) for $\sim 2\frac{1}{2}$ minutes while the upper arm cuffs are repeatedly inflated to venous occlusion pressure (~ 40 mmHg) for ~ 12 seconds and deflated for ~ 4 seconds. This gives about 9 separate plethysmographic recordings for each arm. The first minute or so of each set of recordings needs to be ignored as forearm blood flow adjusts to wrist arterial occlusion. This leaves the last 5 recordings to be used for further analysis (Figure 2.1). Output from the voltage plethysmograph is transferred via an analog-to-digital converter (MacLab 4e, AD Instruments, Hampstead, London) to a personal computer (PowerMac, Apple Computer Incorporated, USA). The data is plotted using Chart software (Chart version 3.2.8; AD Instruments) and analysed using Excel software (Excel version 7.0, Microsoft Corporation, USA).

2.7 Analysis

Plethysmographic recording gives an upward slope during venous occlusion,

the gradient of which is proportional to the rate of blood flow. For the purposes of our studies, we were interested in the effect of drug infusion in the non-dominant arm. The ratio of the gradient of the slope of plethysmographic recording in the infused arm to the gradient of the slope of plethysmographic recording in the non-infused arm is therefore equivalent to the ratio of blood flow between the two arms. The mean of the 5 last plethysmographic recordings is used to reduce the influence of background variation and improve the "signal:noise ratio". The effect of drug infusion is expressed in terms of percentage increase or decrease in blood flow, and is calculated by dividing the ratio of blood flow between the two arms during drug infusion by the ratio of blood flow between the two arms at baseline (Table 2.1). Provided blood pressure remains constant, increases in blood flow represent vasodilation and decreases in blood flow represent vasoconstriction. It is possible to use blood pressure to calculate forearm vascular resistance, but this gives an arbitrary sense of objectivity, and is not recommended. It is also possible to calibrate the equipment so that blood flow in one or both arms can be expressed in absolute units if required. This means preserving the denominator of time and expressing the gradient in mls per 100mls of forearm volume per minute by comparison with a 1% calibration spike (which obviously represents 1 ml per 100mls of forearm volume).

2.8 Blood flow results

The above method is less complicated than it appears. Forearm blood flow, as we have mentioned, is very sensitive to multiple influences such as noise, temperature and discomfort of any sort. Whilst it is still worthwhile controlling these influences, as described above, the method has the strong attraction of factoring all of these

influences out of the equation. In using the ratio of blood flow between the two arms to examine the effect of drug infusion, we are effectively using the non-infused arm as a contemporaneous control. All extraneous influences will influence both arms equally, so that the *only* difference between the two arms is the difference between drug infusion and no drug infusion. In addition, the results are adjusted to take account of any differences at baseline, so that any effect of needle insertion or saline infusion is also factored out of the equation. In a set-up similar to our own, the technique has been shown to be highly reproducible, with an intra-subject variability of 19% between studies (Petrie *et al*, 1998).

2.9 Alternative methods of blood flow measurement

Given the attractions of this method, it is hardly surprising that it is regarded as the "gold standard" of *in vivo* vasomotor assessment (Roberts *et al*, 1986; Benjamin *et al*, 1995; Petrie *et al*, 1998), and over 700 papers using the method have been published over the last 30 years or so. The principle alternatives that have been explored are direct intravascular measurement of blood flow, and external ultrasonic measurement of blood flow. The first of these has the attraction that it seems to be more direct, but it has the considerable disadvantage that it requires much larger more complex intravascular catheters and is therefore more invasive (and potentially more risky). The second has the attraction that it seems to be less invasive, but it has the disadvantage that it cannot measure blood flow directly. Instead, it depends on the measurement of blood flow velocity and arterial diameter individually (by separate ultrasound modalities, 2D and Doppler), and the product of the two to give a measure of volumetric blood flow. This means that two separate sources of error are

multiplied together to give an increased margin of error. It is remarkable how often papers using this method report their results selectively (perhaps because their hypothesis is sustained by one or two out of the measures of blood flow velocity, arterial diameter and volumetric blood flow, but not by the others).

2.10 Measurement of venous capacitance

Venous occlusion plethysmography can also be used to provide a measure of venous capacitance, which is a measure of venous distensibility or venous tone. If the arm cuffs are inflated to venous occlusion pressure and not deflated, forearm size will continue to increase until venous pressure comes to equal the venous occlusion pressure (after which point, venous outflow will resume). Using exactly the same ward set-up and subject set-up described above, the wrist cuffs are inflated to arterial occlusion pressure. Once the plethysmographic recordings have stabilised, after approximately 15 seconds, the upper arm cuffs are inflated to venous occlusion pressure. Once the plethysmographic recordings have plateaued, after approximately 2 minutes, the upper arm cuffs are deflated. Once the plethysmographic recordings have returned to baseline again, after approximately 15 seconds, the wrist cuffs are deflated. The difference between the peak plethysmographic recording and baseline (which can be taken as a mean of the baseline just before venous occlusion and the plateau following relief of venous occlusion) is a measure of venous distensibility, i.e., venous capacitance. This can be expressed in mls per 100 mls of forearm volume by comparison with a 1% calibration spike on the plethysmograph (which obviously represents 1 ml per 100 mls of forearm volume).

2.11 Pulse and blood pressure measurement

Pulse and blood pressure were recorded at regular intervals in all studies by either a manual sphygmomanometer or a semi-automatic sphygmomanometer. This was either applied to the dominant arm over the venous occlusion cuff or to the calf on the same side. It is obviously important not measure blood pressure simultaneously with plethysmographic recordings. Some of the protocols employed allowed time for measurement of blood pressure in the arm between plethysmographic recordings. Some protocols did not leave enough time for this, however, and in these cases the calf was used. We therefore took care to make sure that there was at least 10mmHg agreement between calf blood pressure and brachial blood pressure in these cases.

Table 2.1 **Calculations for Figure 2.1**

Ratio of blood flow in left : right arm

= ratio of gradient of plethysmographic recording in left channel : gradient of plethysmographic recording in right channel

= ratio of height of slope of plethysmographic recording in left channel : height of slope of plethysmographic recording in right channel
e.g., $\delta vlb5 \div \delta vrb5$ for the last baseline recording.

Thus mean ratio of blood flow in left : right arm at baseline = $(\delta vlb1/\delta vrb1 + \delta vlb2/\delta vrb2 + \delta vlb3/\delta vrb3 + \delta vlb4/\delta vrb4 + \delta vlb5/\delta vrb5) \div 5$

& mean ratio of blood flow in left : right arm during drug infusion = $(\delta vld1/\delta vrd1 + \delta vld2/\delta vrd2 + \delta vld3/\delta vrd3 + \delta vld4/\delta vrd4 + \delta vld5/\delta vrd5) \div 5$

Thus ratio of blood flow in the infused arm during drug infusion : blood flow in the infused arm at baseline

= $(\delta vld1/\delta vrd1 + \delta vld2/\delta vrd2 + \delta vld3/\delta vrd3 + \delta vld4/\delta vrd4 + \delta vld5/\delta vrd5) \div (\delta vlb1/\delta vrb1 + \delta vlb2/\delta vrb2 + \delta vlb3/\delta vrb3 + \delta vlb4/\delta vrb4 + \delta vlb5/\delta vrb5)$
which can be expressed as % vasodilation by subtracting 1 and then multiplying by 100%.

To calculate blood flow in absolute units it is necessary to keep the time part of the equation

e.g., $\delta vrb5 \div \delta trb5$ for the last baseline recording in the right arm

Thus mean blood flow in right arm at baseline = $(\delta vrb1/\delta trb1 + \delta vrb2/\delta trb2 + \delta vrb3/\delta trb3 + \delta vrb4/\delta trb4 + \delta vrb5/\delta trb5) \div 5$

& mean blood flow in left arm at baseline = $(\delta vlb1/\delta tlb1 + \delta vlb2/\delta tlb2 + \delta vlb3/\delta tlb3 + \delta vlb4/\delta tlb4 + \delta vlb5/\delta tlb5) \div 5$

Thus mean forearm blood flow at baseline =

$(\delta vrb1/\delta trb1 + \delta vrb2/\delta trb2 + \delta vrb3/\delta trb3 + \delta vrb4/\delta trb4 + \delta vrb5/\delta trb5 + \delta vlb1/\delta tlb1 + \delta vlb2/\delta tlb2 + \delta vlb3/\delta tlb3 + \delta vlb4/\delta tlb4 + \delta vlb5/\delta tlb5) \div 10$

To convert this (which is in mV/s) to ml/min/100ml forearm volume, multiply by 60s and divide by the height of a 1% calibration spike (which corresponds to 1ml/100ml forearm volume)

Figure 2.1 Schematic representation of plethysmographic recordings (blood flow measurement)

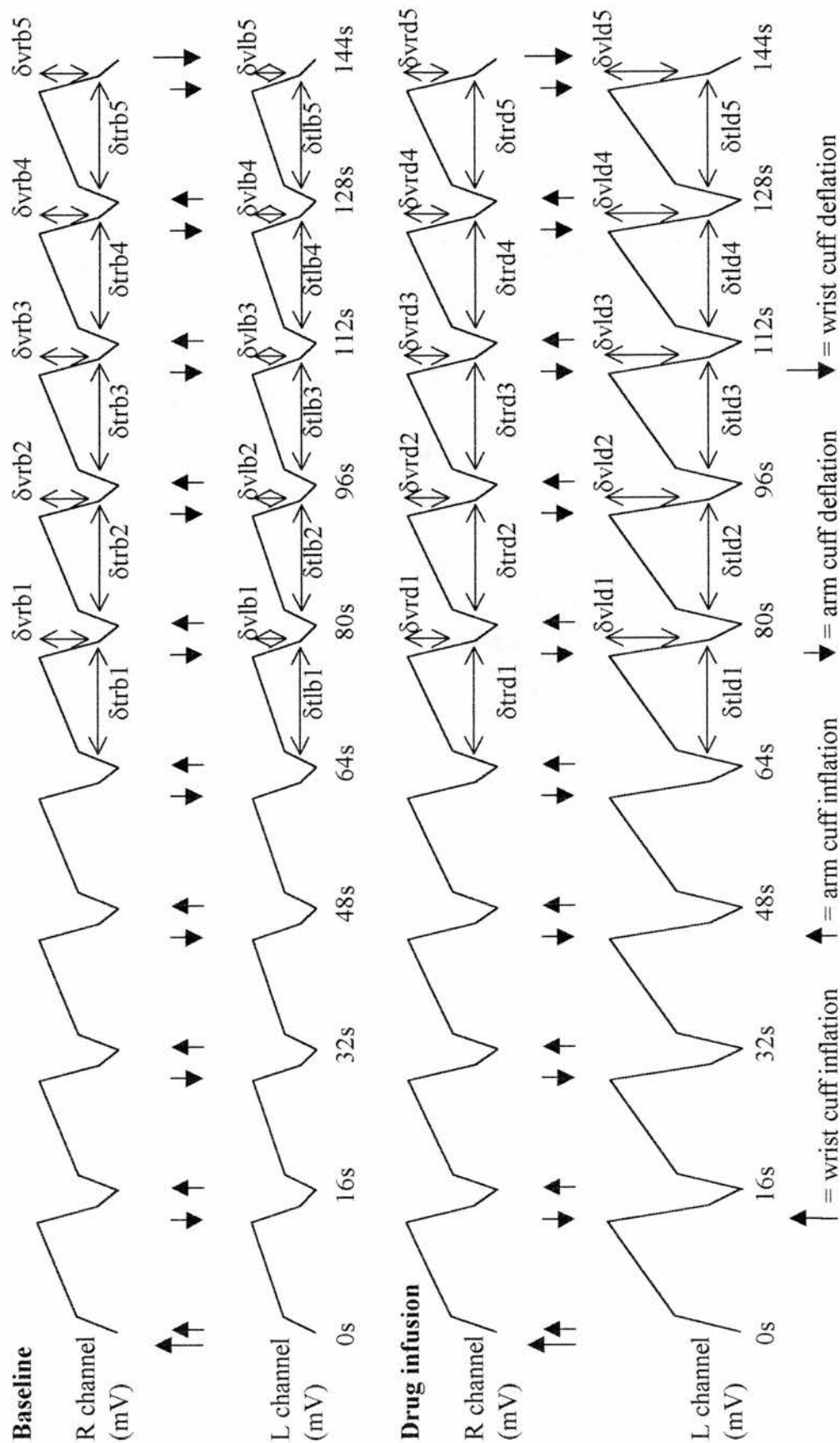
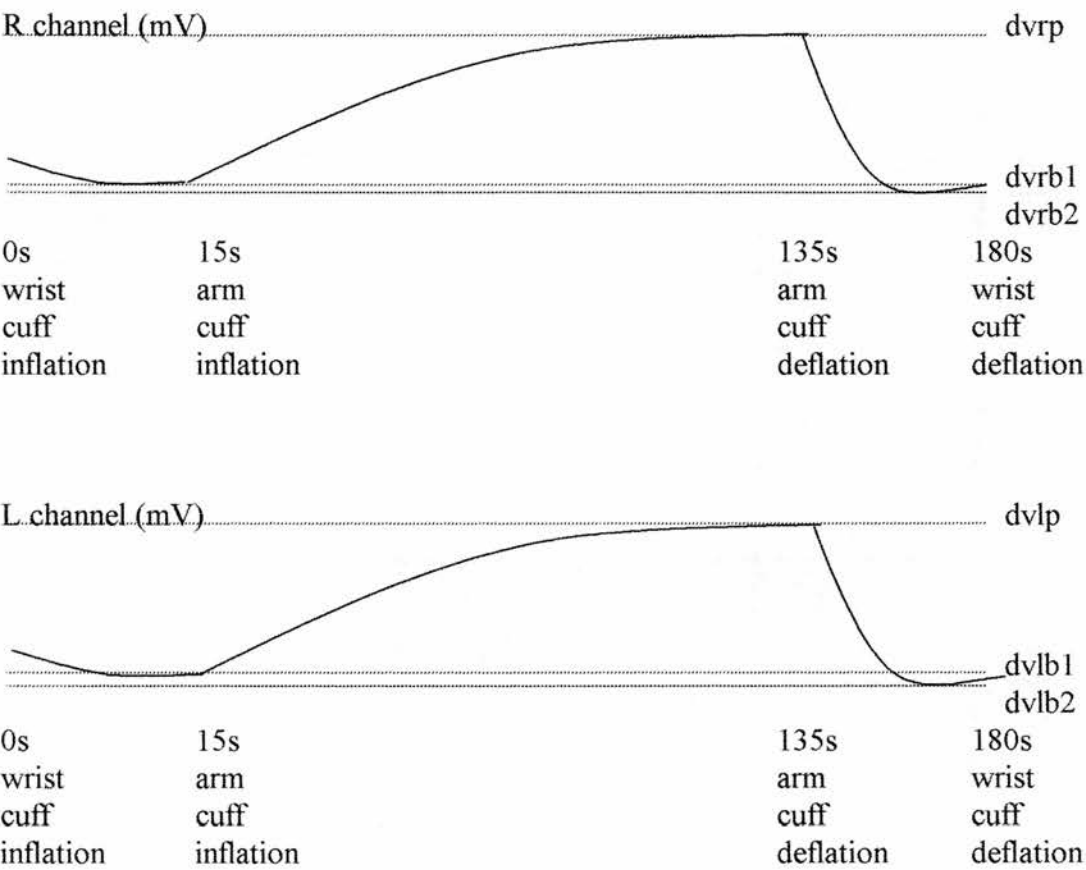


Figure 2.2 Schematic representation of plethysmographic recordings (venous capacitance measurement)



Venous capacitance (right arm) = $dvrp - ((dvrb1 + dvrb2) \div 2)$
Venous capacitance (left arm) = $dvlp - ((dvlb1 + dvlb2) \div 2)$
which both need to be divided by the height of a 1% calibration spike (which corresponds to 1ml/100ml forearm volume) to give venous capacitance in mls per 100mls forearm volume

3 Effect of arachidonic acid in heart failure

3.1 Introduction

Despite over 30 years' study, it remains unclear whether or not low-dose aspirin has clinically significant vascular effects, in addition to its antiplatelet effects. On the one hand, it has been suggested that aspirin is not safe in heart failure (Cleland *et al*, 1995), or that aspirin might counteract the beneficial effects of angiotensin converting enzyme (ACE) inhibitors in heart failure (Hall *et al*, 1992). On the other hand, it has been suggested that aspirin might improve endothelial dysfunction (Husain *et al*, 1998; Noon *et al*, 1998), or that aspirin and ACE inhibitors might have similar modes of action (Jeserich *et al*, 1995). It is important to know the truth, given the widespread use of aspirin in patients with heart failure due to ischaemic heart disease. The absence of any obvious haemodynamic effect of aspirin on its own has necessitated examination of more complex interactions. Unfortunately these studies have failed to provide a consistent answer (Hall *et al*, 1992; Fitzgerald *et al*, 1983; Masotti *et al*, 1979; Heavey *et al*, 1985). The vascular effects of aspirin might be clarified by selective stimulation of vascular endothelium at the point where aspirin exerts its effect.

Aspirin exerts its effects by inhibition of cyclooxygenase, the first enzyme in the pathway that converts arachidonic acid to prostaglandin (Vane *et al*, 1971). Arachidonic acid is liberated by hydrolysis of phospholipid in the cell membrane by phospholipase A₂ (Gelb *et al*, 1994). The effect of exogenous aspirin on endogenous cyclooxygenase might therefore be effectively interrogated by examination of the effect of exogenous aspirin on exogenous arachidonic acid. Arachidonic acid has been shown to be vasodilating in the precontracted dorsal hand vein of healthy

humans *in vivo* (Bhagat *et al*, 1995), but has not otherwise been studied in humans, or at all in heart failure. Furthermore, arachidonic acid has also been shown to have quite opposite effects in different tissues (Sessa *et al*, 1990; Pagano *et al*, 1991), in different species (Sessa *et al*, 1990; Oyekan *et al*, 1991) or under different conditions (Oyekan *et al*, 1991) in animal studies. We therefore sought to validate the effects of arachidonic acid in a different vascular bed in humans *in vivo*, to examine the effects of heart failure on this pathway, and to examine the effects of clinically relevant doses of aspirin on this pathway in heart failure. It has been suggested that lower doses of aspirin may be more "vascular-sparing" than higher doses (Masotti *et al*, 1979). We were keen to examine this in humans rather than animals, *in vivo* rather than *in vitro*, and with real responses of clinically significant blood vessels.

3.2 Methods

3.2.1 Subjects

The study was conducted with the approval of the West Ethics Committee and all subjects and patients gave written informed consent. A group of 10 healthy volunteers with no medical conditions and on no medications was studied (6 male/4 female; mean age 30 ± 4 years). A number of pilot and dose-ranging studies were also conducted in these subjects, in order to establish the safety of intrabrachial arachidonic acid and an appropriate dose for demonstration of its arterial vasomotor effects (if any).

3.2.2 Patients

Fifteen patients with chronic heart failure secondary to left ventricular systolic

dysfunction confirmed by echocardiography were studied (left ventricular ejection fraction <40%). All patients were clinically stable on constant medication for 3 months, with no peripheral oedema or pulmonary congestion, and no uncontrolled hypertension, hypercholesterolaemia or diabetes mellitus. All patients were treated with an ACE inhibitor (enalapril 10mg bd in the majority). All medication apart from aspirin was constant throughout the study. Patient characteristics are summarised in Table 3.1.

3.2.3 Treatment

Healthy volunteers were studied having abstained from all aspirin for 14 days, from alcohol, caffeine and tobacco for 24 hours, and from food for 6 hours. Patients were all studied on three separate occasions :- after 14 days of aspirin 0mg daily; after 14 days of aspirin 75mg daily; and after 14 days of aspirin 300mg daily (having abstained from all other aspirin during that time). The order of study was varied although not formally randomised (the majority were habitually on aspirin 75mg and were therefore studied in the order: 75mg; 0mg; 300mg). Patients took their usual medications including aspirin as directed 6 hours before attendance on the day of study, having abstained from alcohol, food, caffeine and tobacco as above. The study design is summarised in Figure 3.1.

3.2.4 Measurements

Forearm blood flow was measured by the technique of venous occlusion plethysmography as described in chapter 2. Plethysmographic recordings were made every 5 minutes, and pulse and blood pressure were manually recorded in the non-

infused arm every 10 minutes.

3.2.5 Drugs

Sodium arachidonate (5 mg per vial) stored under nitrogen was obtained from Sigma. Vials were stored at -20°C , and a single vial was used for each study, used within 2 hours and discarded thereafter. Sodium arachidonate (5mg) was dissolved in 154 μl absolute alcohol, filtered through a 1 μm filter, and diluted with physiological saline to a final concentration to be infused at 1 ml/min. In the first few pilot studies the doses chosen were 200 pmol/min, 2 nmol/min and 20 nmol/min (as in the vein studies of Bhagat *et al*, 1995), each for 20 minutes. When further dose-ranging studies were required the doses arbitrarily chosen were 20 nmol/min for 60 minutes, and 83, 112, 116, 150, 200 and 225 nmol/min each for 30 minutes. The dose finally settled on was 112 nmol/min for 45 minutes (the total dose was therefore 5 μmol).

3.2.6 Data analysis

All results are expressed as mean values with standard errors. Because repeated serial measurements were made in every study, comparison between studies was performed using summary measures, i.e., mean response over the 45 minute infusion period and peak response during the 45 minute infusion period, to avoid making multiple comparisons on dependent variables (Matthews *et al*, 1990). Summary measures were then compared using two-tailed paired t tests with Bonferroni adjustments as required for multiple comparisons. Differences were considered significant at a value of $p < .05$.



3.3 Results

3.3.1 General effects

Local infusion of arachidonic acid caused no adverse or systemic effects, and subjects and patients did not report any discomfort. Pulse rate (Figure 3.2), blood pressure (Figure 3.3), and forearm blood flow in the non-infused forearm did not change significantly on any of the studies, and there were no significant differences in baseline pulse rate (Figure 3.2), blood pressure (Figure 3.3) or forearm blood flow between any of the studies.

3.3.2 Pilot and dose-ranging studies

The initial three studies were closely modelled after the vein studies of Bhagat *et al* (1995), as these were the only studies with arachidonic acid *in vivo* in humans in the literature at the time. Arachidonic acid was therefore infused at 200 pmol/min, 2 nmol/min and 20 nmol/min, albeit for 20 minutes each rather than the 5 minutes each of the earlier study. When there was no obvious effect at any of these doses, we tried a 60 minute infusion of 20 nmol/min in three further studies. When there was no obvious effect of this either, we tried 30 minute infusions of 83, 112, 116, 150, 200 and 225 nmol/min. The lower three doses produced modest vasodilation, and the higher three doses produced dramatic vasodilation, but in two out of the three cases, this was complicated by occlusion of the intrabrachial cannula. We therefore settled on a 45 minute infusion of 112 nmol/min as a compromise. This meant that the total dose delivered was 5000nmol, which compares favourably with the total dose of 100nmol delivered in the highest dose vein study (since the rate of blood flow in the brachial artery is approximately 50 times that in the dorsal hand vein it is necessary to

deliver up to 50 times the dose of any drug to have an equipotent effect).

3.3.3 Effect of arachidonic acid infusion

Local infusion of 112 nmol/min of arachidonic acid for 45 minutes produced progressive and incremental forearm vasodilation throughout the infusion period. Figure 3.4 shows the effects of 112 nmol/min of arachidonic acid for 45 minutes in healthy volunteers. The mean response was a dilatation of $33\pm6\%$ and the peak response was a dilatation of $64\pm10\%$.

3.3.4 Effect of heart failure on response to arachidonic acid

There was no significant difference in vasodilation to arachidonic acid between healthy volunteers and patients with heart failure with no aspirin pre-treatment. Neither mean vasodilation ($33\pm6\%$ versus $28\pm7\%$) (Figure 3.5) nor peak vasodilation ($64\pm10\%$ versus $55\pm11\%$) (Figure 3.6) was significantly different.

3.3.5 Effect of aspirin on response to arachidonic acid infusion

Mean vasodilation was reduced by 55% following pre-treatment with 75mg aspirin ($13\pm8\%$ versus $28\pm7\%$) and by 59% with 300mg aspirin ($12\pm7\%$ versus $28\pm7\%$) (Figure 3.5). Peak vasodilation was reduced by 35% following pre-treatment with 75mg aspirin ($36\pm12\%$ versus $55\pm11\%$) and by 45% with 300mg aspirin ($30\pm9\%$ versus $55\pm11\%$) (Figure 3.6). The differences between no aspirin pre-treatment and aspirin pre-treatment were significant regardless of dose, and there was no significant difference between the two different doses of aspirin.

3.4 Discussion

We have shown that arachidonic acid causes vasodilation in the forearm of humans *in vivo*, that this response is unaffected by heart failure, that in patients with heart failure this response is inhibited by aspirin, and that this inhibition is apparent with a dose as low as 75mg once daily. The first of these findings is novel but perhaps not unexpected, the second is novel and quite unexpected, the third is novel although very much as expected, and the fourth is novel, quite unexpected and has potential implications for vascular biology (and even more importantly for clinical medicine).

3.4.1 Safety of arachidonic acid

We have no way of knowing why the higher doses of arachidonic acid were complicated by occlusion of the intrabrachial cannula. Obviously we can't help suspecting the possibility of platelet activation and aggregation at the needle tip. Although the concentrations of arachidonic acid used would have been enough to cause platelet activation *in vitro*, we had hoped to avoid any possibility of this *in vivo* because of the rate of blood flow within the brachial artery and rapid dissipation of the infused solution. We avoided the use of these higher doses in further studies.

3.4.2 Vasodilation by arachidonic acid

This is the first demonstration that arachidonic acid is vasodilating in arteries in humans *in vivo*. Although we had no control infusion for arachidonic acid and it is therefore possible that the effect was due to vehicle (i.e., a very low concentration of ethanol), this is unlikely, as ethanol is well-recognised to be a vasoconstrictor when

directly applied to blood vessels (Hayes *et al*, 1988). As previously noted, it has previously been demonstrated that arachidonic acid is vasodilating in veins in humans *in vivo* (Bhagat *et al*, 1995). As previously noted also, it has been demonstrated that the response to arachidonic acid is extremely heterogeneous between different tissues, different species and different conditions in animals (Sessa *et al*, 1990; Pagano *et al*, 1991; Oyekan *et al*, 1991). The demonstration that arachidonic acid is indeed vasodilating in arteries as well as in veins confirms the potential importance of this pathway in humans *in vivo*. It also suggests the potential importance of this pathway in heart failure and the potential importance of modulation of this pathway in this syndrome. Notwithstanding, it is important to remember that the effects of exogenous administration of an agonist do not necessarily tell us very much about the endogenous activity of the same agonist, if any.

3.4.3 Effect of heart failure on response to arachidonic acid

It is surprising that the response to arachidonic acid appeared to be so little different between healthy volunteers and patients with heart failure, as it has previously been reported that cyclooxygenase gene expression is down-regulated in heart failure (Smith *et al*, 1996). There is no doubt that our patients had clinically well-characterised heart failure, but it would have been desirable to have had more objective information about them at baseline, such as an assessment of their degree of neuroendocrine activation. It would clearly have been preferable to have age-matched controls, but given that there was so little difference between our controls and our patients with heart failure, it seems unlikely that age-matched controls would have been much different from either, but we cannot know this. Indeed, given that there

was such an age-difference, it is perhaps even less likely that the small non-significant difference shown was due to heart failure. It is equally possible that there is some deficiency in heart failure patients which was compensated for by their ACE inhibitor treatment, but we cannot know this either. It is clear that there is some heterogeneity of vasodilator responses in heart failure. It is clear that the response to acetylcholine or methacholine is reduced and that the response to nitroglycerin or sodium nitroprusside is not (Kubo *et al*, 1991). At the same time, there is some controversy surrounding the response to substance P (Hirooka *et al*, 1992; Lindsay *et al*, 1996), and surprisingly little information on the response to bradykinin (Cheng *et al*, 1998; Su *et al*, 1998). Our findings suggest that at least some aspects of vascular prostanoid production and activity may be unimpaired in patients with treated heart failure, but this is clearly deserving of further study.

3.4.4 Effect of aspirin on response to arachidonic acid

The most striking finding of our study was that vasodilation to arachidonic acid was significantly blocked (by over half) by a dose of aspirin as small as 75mg once daily in patients with heart failure. It would have been preferable to make this comparison on the basis of randomised double-blind treatment, but the magnitude of the reduction makes it unlikely that it is simply a result of bias. This contradicts the widely held view that such small doses of aspirin can be regarded as "platelet-specific" or "vascular-sparing". In fact, this may be consistent with the majority of previous studies in this field, but it is much more clinically relevant because we chose to examine the chronic (14-day) effects of the doses in common use for cardioprotection, something which previous studies have failed to do.

3.4.5 Comparison with effects of aspirin on prostacyclin

The earliest studies with any relevance to the vascular effects of aspirin measured the levels of thromboxane and prostacyclin, focusing on preserved levels of prostacyclin as a measure of preserved endothelial cyclooxygenase activity. The results of such studies are not particularly consistent. At least three studies have shown preserved prostacyclin, with a single dose below 75mg (Hanley *et al*, 1981), with 7 days of less than 75mg (Patrignani *et al*, 1982), and with a single dose of well above 75mg (Masotti *et al*, 1979). At the same time two studies have shown suppressed prostacyclin with 7 days of doses well below 75mg (Fitzgerald *et al*, 1983; Weksler *et al*, 1985). So far as it is possible to collate these results, there appears to be a threshold for suppression of prostacyclin by aspirin which lies somewhere between 20mg and 250mg, with some evidence of a lower threshold for repeated doses than for single doses. The results of such studies cannot really be taken as evidence of an overall vascular effect of aspirin one way or the other, because they did not measure any sort of vascular activity directly. Furthermore, none of these studies was conducted in patients with heart failure, all were in normals, hypertensives or coronary patients.

3.4.6 Comparison with effects of aspirin on bradykinin

More recently, a number of studies have focused on the effect of aspirin on the response to bradykinin. Bradykinin is a potent vasodilator in its own right, which clearly also stimulates prostacyclin production (Barrow *et al*, 1986). There have been a number of studies that have looked at the effects of aspirin on the response to bradykinin, both in terms of vasodilation and in terms of prostacyclin production. The

results of these studies have been a bit more consistent. Three studied the effect of a single dose of 600mg and showed blockade of prostacyclin but not vasodilation (Heavey *et al*, 1985), no effect on vasodilation (Benjamin *et al*, 1989), and blockade of prostacyclin (Ritter *et al*, 1989), respectively. Two more studied the effect of repeated doses of 75mg, for 4 days (Clarke *et al*, 1991), and 14 days (Keimowitz *et al*, 1993), and both showed blockade of prostacyclin. Despite the consistency of these results, the obvious discrepancy between suppression of prostacyclin production and vasodilation calls into question the relevance of prostacyclin production to the vasodilator effects of bradykinin, or indeed to the vasodilator effects of any mediator.

3.4.7 Comparison with effects of aspirin on ACE inhibitors

Most recently a number of studies have attempted to look directly at the interaction between aspirin and ACE inhibitors and again the results have been fairly inconsistent. A single dose of aspirin 350mg counteracted some but not all of the haemodynamic effects of a single dose of enalapril 10mg (Hall *et al*, 1992). A single dose of aspirin 236mg had no effect on the haemodynamic effects of a single dose of captopril 25mg (van Wijngaarden *et al*, 1994). A single dose of aspirin 500mg prevented the apparent improvement in endothelial function from intra-arterial enalaprilat (Nakamura *et al*, 1994). Aspirin 250mg had no effect on the neurohormonal or haemodynamic effects of enalapril 5-10mg either overnight or after 4 weeks (Baur *et al*, 1995). Aspirin 325mg od counteracted the 15 day improvement in pulmonary diffusion from enalapril 10mg bd (Guazzi *et al*, 1997). Aspirin 325mg od for 7 days (but not ticlopidine) counteracted some but not all of the haemodynamic effects of a single dose of enalapril 10mg (Spaulding *et al*, 1998). Neither aspirin nor

ifetroban (a thromboxane receptor antagonist) had any effect on the effect of 6 weeks of enalapril on forearm blood flow (Katz *et al*, 1999).

3.4.8 Effects of aspirin on arachidonic acid

The only other experience of the effect of aspirin on vasodilation stimulated by exogenous arachidonic acid in humans was reported by Bhagat *et al* (1995). These authors found that a single large dose of oral aspirin (1g) inhibited arachidonic acid-induced venodilation 2 hours later, but low-dose aspirin (75mg) did not. Whether the difference from our results reflects a difference in the sensitivity of arteries and veins, a difference between acute and chronic dosing, a difference between healthy subjects and heart failure, an interaction with ACE inhibitors, or some combination of these factors, we do not know. Any of these considerations would be ripe for further investigation.

3.4.9 Summary

We chose to look at the effects of chronic daily dosing with aspirin, and we chose to compare the effects of the highest dose in common use for cardioprotection (300mg) with the lowest dose in common use for cardioprotection (75mg). Whilst our findings really only apply to the time window in which they were conducted (6 - 8 hours after the last dose of aspirin) this implies a significant portion of the day, (a quarter to a third at the very least), during which even very low dose aspirin has detectable potentially detrimental effects. There are lower doses of aspirin which achieve adequate inhibition of platelet cyclooxygenase (Patrono *et al*, 1985), and which might not affect vascular arachidonic acid metabolism. There are also other

formulations of aspirin (Clarke *et al*, 1991), or routes of administration of aspirin (Keimowitz *et al*, 1993; Pedersen *et al*, 1984; Cerletti *et al*, 1986), which might do the same. None of these is in routine clinical practice, however, and we sought to make our findings as clinically relevant as possible. Although it is possible that fourteen days is not long enough to observe the full vascular profile of aspirin (which might be more complex), it is longer than the treatment in any other comparable study. Our findings lead us to suspect that the reason that vasodilation was so sensitive to even a very low dose of aspirin may be because of the length of treatment in our study.

Table 3.1 Patient Characteristics

<i>Patient Characteristic</i>	
Sex, n	13 M/2 F
Age, y (mean±SD)	69±7
Primary diagnosis IHD, n	14
IDC, n	1
NYHA functional class II, n	11
III, n	4
LVEF, % (mean±SD)	21±7
Current smoker, n	1
On ACE inhibitor, n	15
Enalapril 10mg bd, n	12
Aspirin, n	12
Diuretic, n	10
Nitrate, n	6
Statin, n	6
Beta blocker, n	5
Calcium antagonist, n	3
Amiodarone, n	2
Oral hypoglycaemic	2
Digoxin, n	1

IHD = ischaemic heart disease; IDC = idiopathic dilated cardiomyopathy; NYHA = New York Heart Association; LVEF = left ventricular ejection fraction

Figure 3.1 Study design

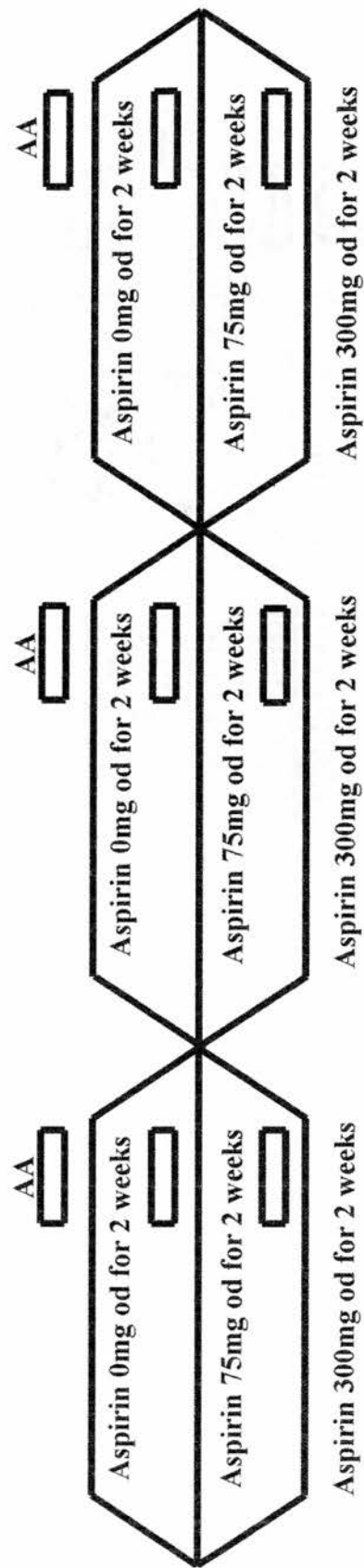


Figure 3.2 Heart rate during arachidonic acid infusion in 15 patients according to aspirin pre-treatment (p=ns for all comparisons)

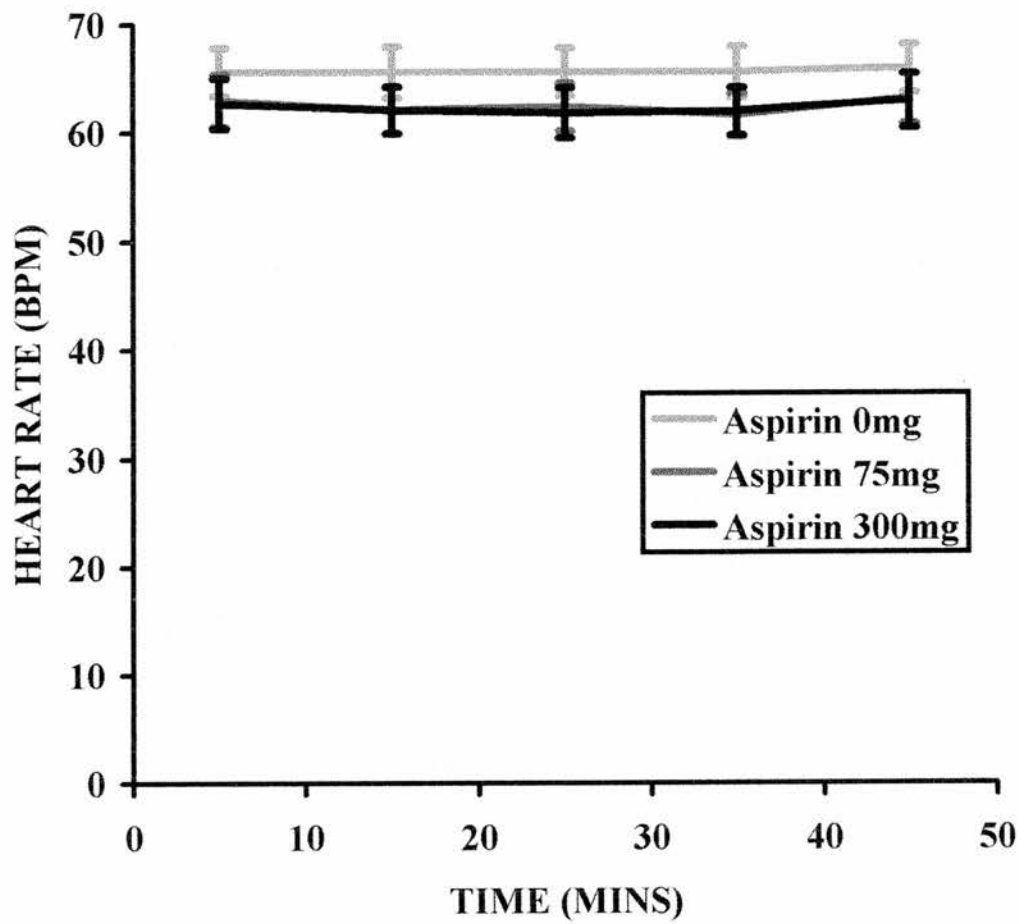


Figure 3.3 Mean blood pressure during arachidonic acid infusion in 15 patients according to aspirin pre-treatment (p=ns for all comparisons)

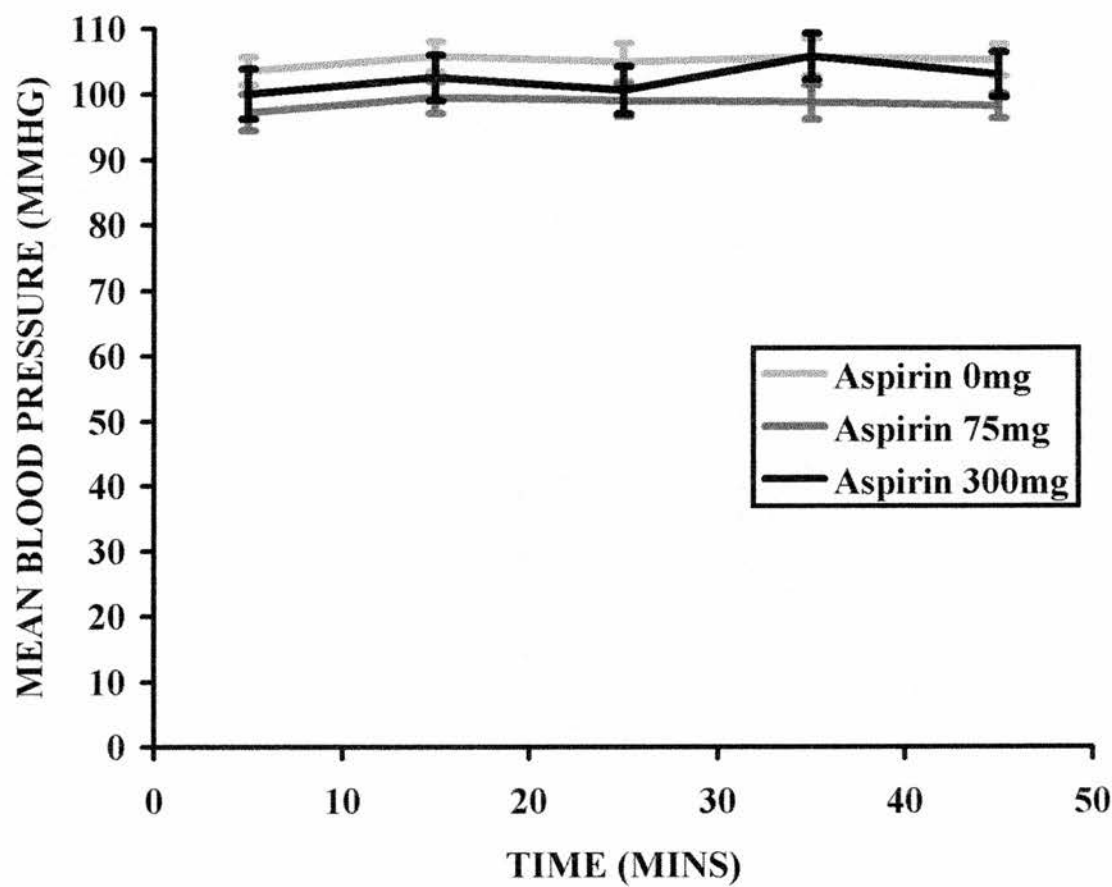


Figure 3.4 Effect of brachial artery infusion of arachidonic acid 112
nmol/min for 45 minutes in 10 controls

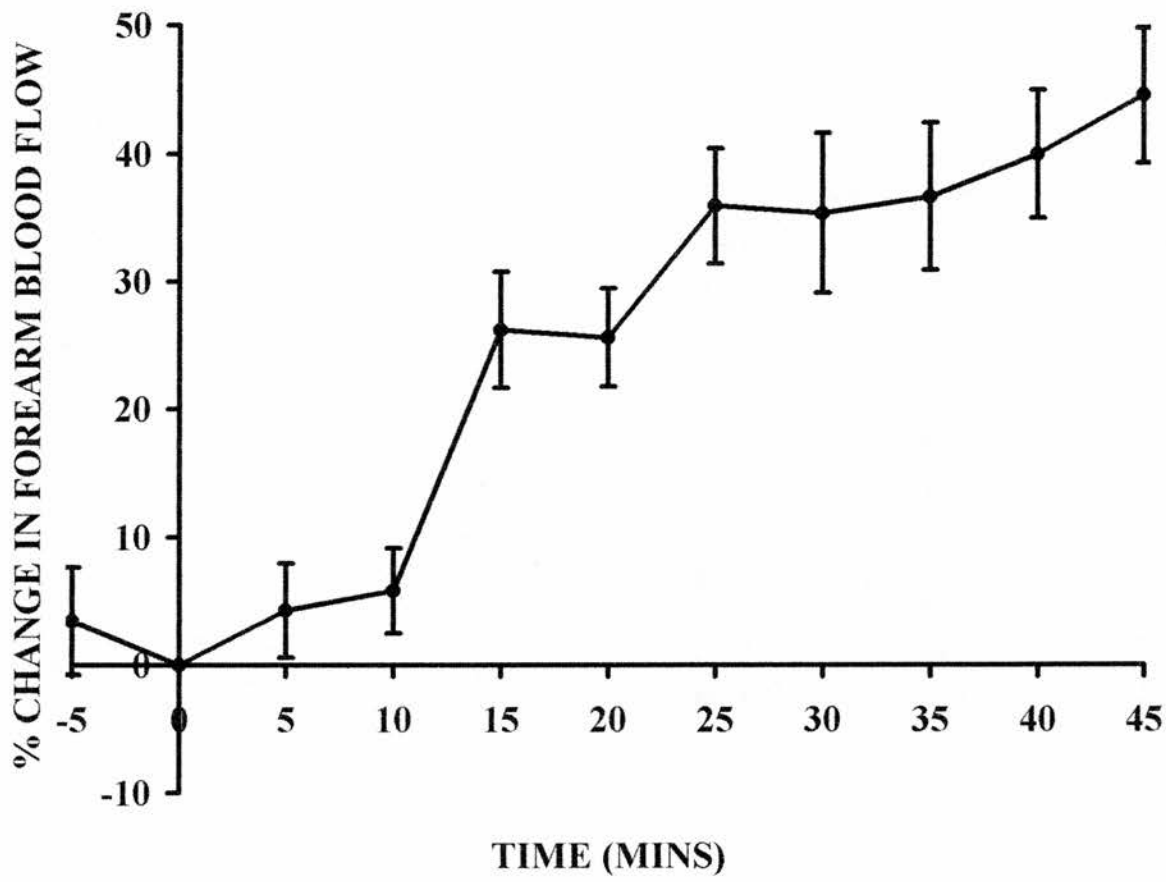


Figure 3.5 Mean vasodilation according to aspirin pre-treatment (control versus aspirin 0mg, p=ns; aspirin 75mg versus aspirin 0mg, p=.0025; aspirin 300mg versus aspirin 0mg, p=.021; aspirin 75mg versus aspirin 300mg, p=ns)

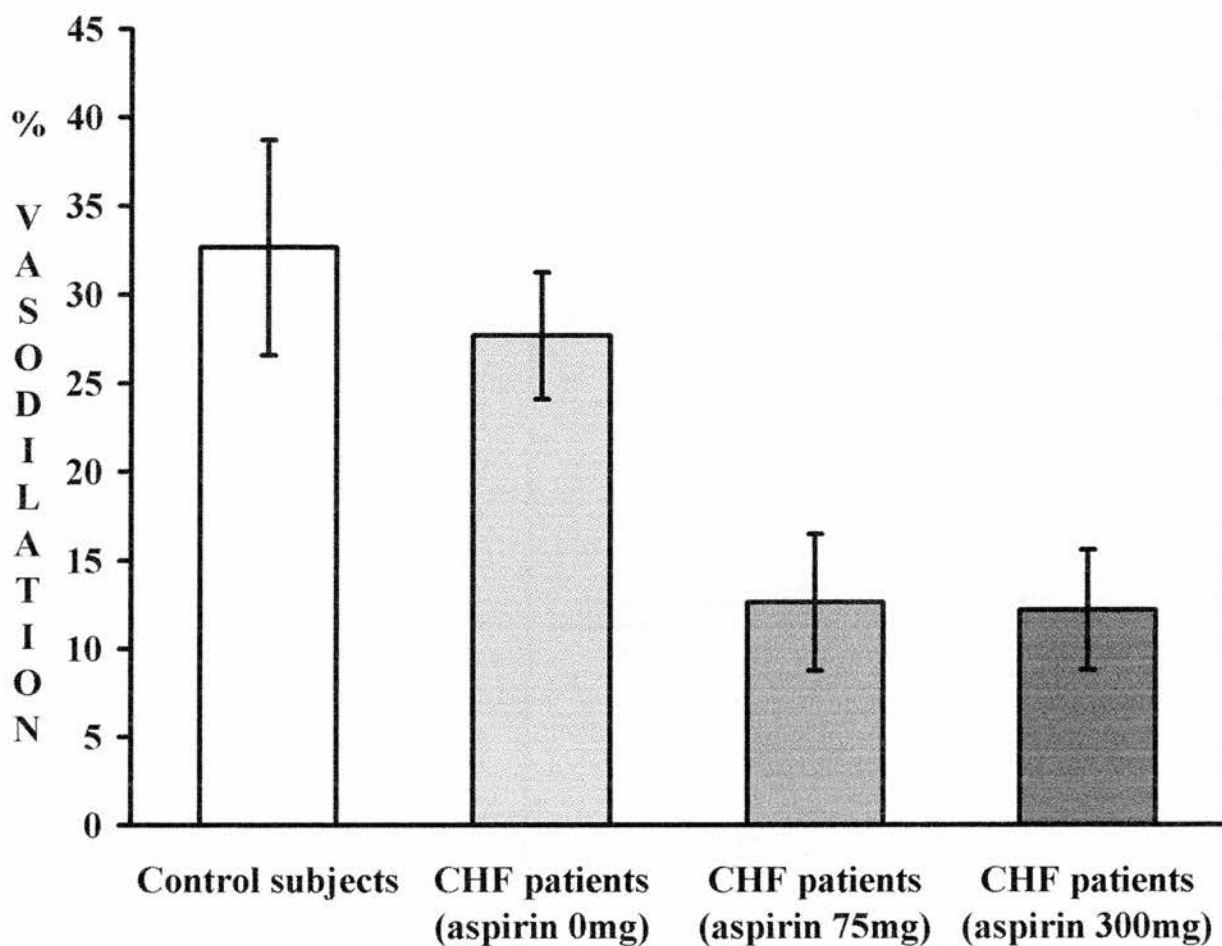
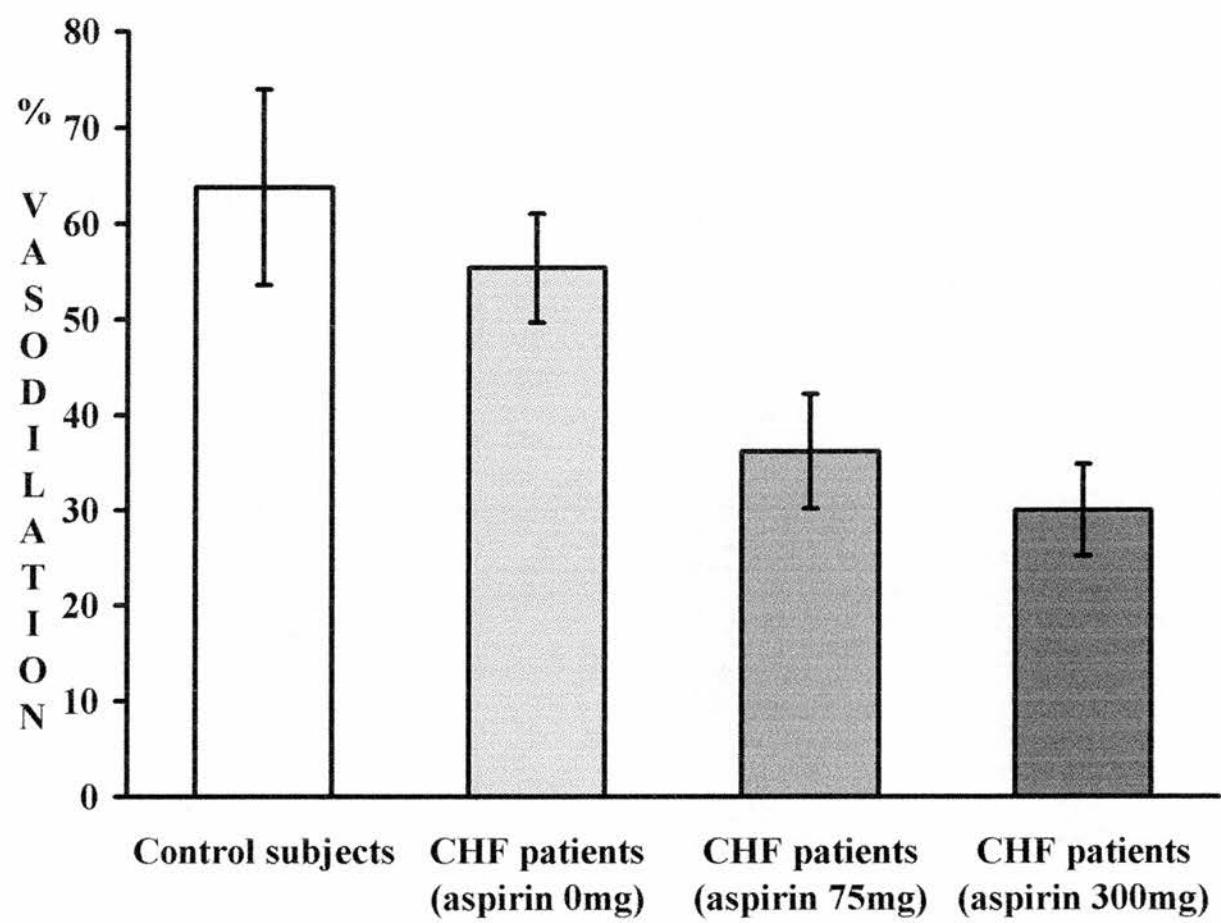


Figure 3.6 Peak vasodilation according to aspirin pre-treatment (control versus aspirin 0mg, p=ns; aspirin 75mg versus aspirin 0mg, p=.077; aspirin 300mg versus aspirin 0mg, p=.006; aspirin 75mg versus aspirin 300mg, p=ns)



4.1 Introduction

Intravenous furosemide is commonly administered to patients with acute heart failure to relieve pulmonary congestion through diuresis. However, it has long been recognised that symptomatic relief occurs in these patients before diuresis has had time to occur (Weinstein & Solis-Gil, 1966; Biagi & Bapat, 1967; Bhatia *et al*, 1969). It has also long been recognised that this effect may be a consequence of a venodilator effect which precedes the diuretic effect (Dikshit *et al*, 1973). It is fairly well-established that the venodilator response to furosemide is inhibited by the cyclooxygenase inhibitor indomethacin, so it is usually assumed that this venodilation is brought about through the release of local prostanoids (Johnston *et al*, 1983; Pickkers *et al*, 1997). Our hypothesis was that aspirin might also inhibit the acute venodilator response to furosemide in patients with chronic heart failure. The present study was designed to determine whether or not the doses of aspirin shown to block the arterial vasodilator response to arachidonic acid in chapter 3 (75mg and 300mg daily) also affect the venodilator response to furosemide. We also studied the immediate venodilator response to sublingual glyceryl trinitrate (GTN) to set the venodilator effect of furosemide in a more clinically familiar context.

4.2 Methods

4.2.1 Patients

The study was conducted with the approval of the West Ethics Committee and all patients gave written informed consent. Eleven patients with chronic heart failure secondary to left ventricular systolic dysfunction confirmed by echocardiography (left ventricular ejection fraction <40%) were studied in each study. All patients were

clinically stable on constant medication for 3 months, with no peripheral oedema or pulmonary congestion, and no uncontrolled hypertension, hypercholesterolaemia or diabetes mellitus. All patients were treated with an ACE inhibitor (enalapril 10mg bd in the majority). All medication apart from aspirin was constant throughout the first study and all medication was constant in the second study. Patient characteristics for the two studies are summarised in Table 4.1.

4.2.2 Randomisation

Patients in the first study were randomised to double-blind cross-over treatment with aspirin 75mg for 14 days, aspirin 300mg for 14 days and paracetamol 120mg for 14 days (as a non-vasoactive placebo). At the end of each 14 day study period, patients attended for venous occlusion plethysmography and the following day crossed over to the next randomly assigned drug. During each 14 day period patients abstained from all other aspirin, aspirin-containing or aspirin-like medications. Patients took their usual medication including aspirin/paracetamol 6 hours before attendance on the day of study. Patients abstained from alcohol, tobacco and caffeine for 24 hours before each study and attended fasted for 6 hours. Patients were instructed to withhold their diuretic therapy on the day of study, but to take all other medication as normal. Patients in the second study were not randomised but were invited to take their usual aspirin therapy as prescribed and were instructed to withhold any nitrate therapy on the day of study, but to take all other medication as normal. The study design is summarised in Figure 4.1.

4.2.3 Treatment

In the first study, 20mg of furosemide (Antigen Pharmaceuticals, Rosecrea, Co Tipperary, Ireland) was administered over the course of 30 seconds through a 20G intravenous cannula (Biovalve, Vygon, USA) sited in the forearm. The cannula was then flushed with a further 5ml of physiological saline solution (Baxter Healthcare Corporation, USA). In the second study, 400µg of GTN (Lipha Pharmaceuticals, France) was administered sublingually.

4.2.4 Measurements

Forearm blood flow and venous capacitance were measured in both arms by the technique of venous occlusion plethysmography as described in chapter 2. Plethysmographic recordings were made at 5 minute intervals for 20 minutes before furosemide/GTN administration and at 5 minute intervals for 20 minutes after furosemide/GTN administration. Pulse rate and blood pressure were recorded at 5 minute intervals throughout with a semi-automatic sphygmomanometer (Dinamap Plus 8700; Johnson & Johnson, USA) and a leg cuff wrapped round the right calf.

4.2.5 Data analysis

All results are expressed as mean values with standard errors. Because repeated serial measurements were made in every study, comparison between studies was performed using a summary measure, i.e., mean response over the 20 minutes following furosemide/GTN administration infusion, to avoid making multiple comparisons on dependent variables (Matthews *et al*, 1990). Summary measures were then compared using two-tailed paired t tests. Differences were considered

significant at a value of $p < .05$.

4.3 Results

4.3.1 General effects

Furosemide and GTN administration caused no adverse or obvious systemic effects, and patients did not report any discomfort. Pulse rate and blood pressure did not change significantly during any of the studies. There were no significant differences in baseline pulse rate, blood pressure, forearm blood flow or venous capacitance between any of the studies.

4.3.2 Effect of furosemide/GTN on forearm blood flow

Forearm blood flow was not significantly affected by the administration of intravenous furosemide on any of the three study days. Similarly, forearm blood flow was not significantly affected by the administration of sublingual GTN.

4.3.3 Effect of furosemide/GTN on forearm venous capacitance

In the placebo study there was a $2.25 \pm 1.82\%$ increase in forearm venous capacitance after furosemide administration. In the aspirin 300mg study there was a $3.77 \pm 1.82\%$ fall in venous capacitance after furosemide administration ($p < .0001$ versus placebo). In the aspirin 75mg study there was a $1.24 \pm 1.67\%$ fall in venous capacitance after furosemide administration ($p < .01$ versus placebo; $p = \text{ns}$ versus aspirin 300mg) (Figure 4.2). Glyceryl trinitrate caused a $2.11 \pm 1.91\%$ increase in forearm venous capacitance (Figure 4.3). This was not significantly different from the increase observed after intravenous furosemide in the placebo study ($p = 0.07$).

4.4 Discussion

Furosemide caused an increase in forearm venous capacitance in our patients. This effect has not been described in patients with chronic heart failure before. Previously venodilation has only been described in patient with acute heart failure secondary to myocardial infarction (Dikshit *et al*, 1973) and salt depleted volunteers (Bhatia *et al*, 1969; Johnson *et al*, 1983, 1984, 1985, 1986; Passmore *et al*, 1989). Our data support the hypothesis that furosemide has a potentially beneficial haemodynamic effect before diuresis has time to occur and effect any haemodynamic change itself. In the absence of a control infusion for furosemide, however, we cannot be absolutely certain that the effect of furosemide on its own was significant. This is in marked contrast to the effect of aspirin on the response to furosemide, which was controlled for.

4.4.1 Prostaglandins and venodilation

A daily dose of 75mg and 300mg of the cyclooxygenase inhibitor aspirin inhibited the venodilator effect of furosemide in our study. This observation serves as a compelling validation of our results in chapter 3, and provides further evidence that prostaglandins are involved in the mechanism of this vascular action of furosemide (MacKay *et al*, 1984). What is not clear, however, is precisely how inhibition of prostaglandin synthesis inhibits venodilation. The most obvious explanation is that aspirin inhibits the local production of prostaglandins, which provide the dilatory stimulus to veins. Whether these are released directly in response to the action of furosemide on the veins (Pickkers *et al*, 1997) or indirectly in response to the action of angiotensin II on the venous endothelium (Gimbrone *et al*, 1975) is unknown. If

the latter is the mechanism, aspirin could block it at two sites. Aspirin may block furosemide induced renal renin release, which is prostaglandin dependent (MacKay *et al*, 1984; Goldiner *et al*, 1981; Tan *et al*, 1977) and hence, subsequent generation of angiotensin II. Alternatively, aspirin could block the direct effect of angiotensin II on veins, presumably mediated by cyclooxygenase.

4.4.2 Angiotensin II and venodilation

Involvement of angiotensin II in the process of venodilation (Johnston *et al*, 1983) would however seem unlikely in our patients. It has been previously reported that beta-blocker therapy inhibits the acute renin release produced by intravenous furosemide (Johnstone *et al*, 1985), and six of the eleven patients in our study were taking a beta-blocker as part of their heart failure therapy. Furthermore, all eleven patients in the furosemide study were taking an ACE inhibitor, yet they still displayed venodilation in response to furosemide.

4.4.3 Direct effects of furosemide

A direct effect of furosemide in veins may be more likely and has previously been described by Pickkers *et al* (1997). In their study furosemide was found to have a direct dilatory effect on dorsal hand veins of healthy volunteers. The effect was independent of nitric oxide, and the concentration of furosemide in the veins was far less than the supra-therapeutic concentrations which are needed to inhibit the chloride-dependent Na-K cotransport system (Ellory & Stewart, 1982). Instead, the authors found that the effect of furosemide was blocked by cyclooxygenase inhibition (with indomethacin) and was therefore deemed to be prostaglandin dependent. This

direct action of furosemide has not been reported by all authors (Harada *et al*, 1996). Differences in the venoconstrictor used and the percentage of pre-constriction of the veins before furosemide was administered could, however, account for this discrepancy.

4.4.4 Effect of furosemide on endothelium

Further support for the hypothesis that furosemide stimulates prostaglandin release through an action on the venous endothelium comes from an *in vitro* study by Liguori *et al* (1999). These authors studied the effects of therapeutic concentrations of furosemide on cultures of human umbilical vein endothelium. They reported that secretion of prostacyclin, measured indirectly by 6-keto-prostaglandin $F_{1\alpha}$ production, increased within 5 minutes of exposure of the culture to furosemide. Secretion was maximal at approximately 15 minutes and was maintained for 20 to 30 minutes. This time-scale for the release of prostacyclin fits well with our findings and those of others that the venodilator effect of furosemide occurs within 15 to 20 minutes of drug administration *in vivo*.

4.4.5 Summary

We have demonstrated that venodilation occurs in patients with chronic heart failure in the minutes following the administration of a 20mg intravenous dose of furosemide. This is obviously potentially advantageous, even more so in the clinical scenario of acute heart failure. Our observation that the venodilation could be inhibited by both high and low dose aspirin further questions the use of aspirin in patients with chronic heart failure, over and above the results of chapter 3.

Table 4.1 Patient Characteristics

<i>Patient Characteristic</i>	<i>Furosemide Study</i>	<i>GTN Study</i>
Sex, n	10M/1F	10M/1F
Age, y (mean±SD)	70±7	70±7
Primary diagnosis IHD/IDC, n	9/2	9/2
NYHA functional class II/III, n	6/5	6/5
LVEF, % (mean±SD)	25±8	25±8
Current smoker, n	1	1
On Aspirin, n	10	9
Diuretic, n	8	8
ACE inhibitor, n	11	11
Enalapril 10 mg bd, n	9	9
Beta blocker	4	6
Statin	5	4
Nitrate	3	3
Digoxin	2	1
Amiodarone	2	2
Calcium antagonist	1	1

IHD = ischaemic heart disease; IDC = idiopathic dilated cardiomyopathy; NYHA = New York Heart Association; LVEF = left ventricular ejection fraction

Figure 4.1 Study design

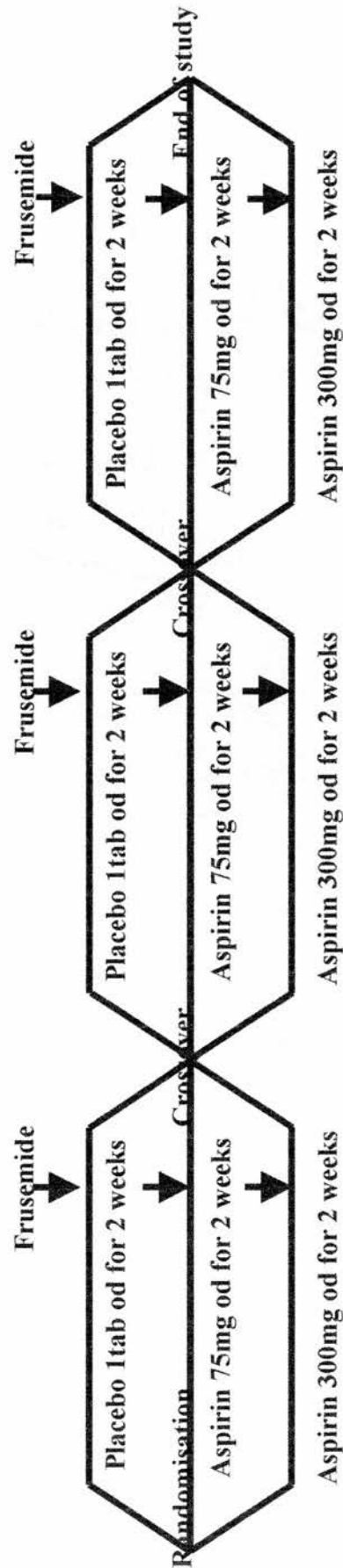


Figure 4.2 Effect of furosemide on venous capacitance according to aspirin pre-treatment (aspirin 75mg versus placebo, $p<.01$; aspirin 300mg versus placebo, $p<.0001$; aspirin 75mg versus aspirin 300mg, $p=ns$)

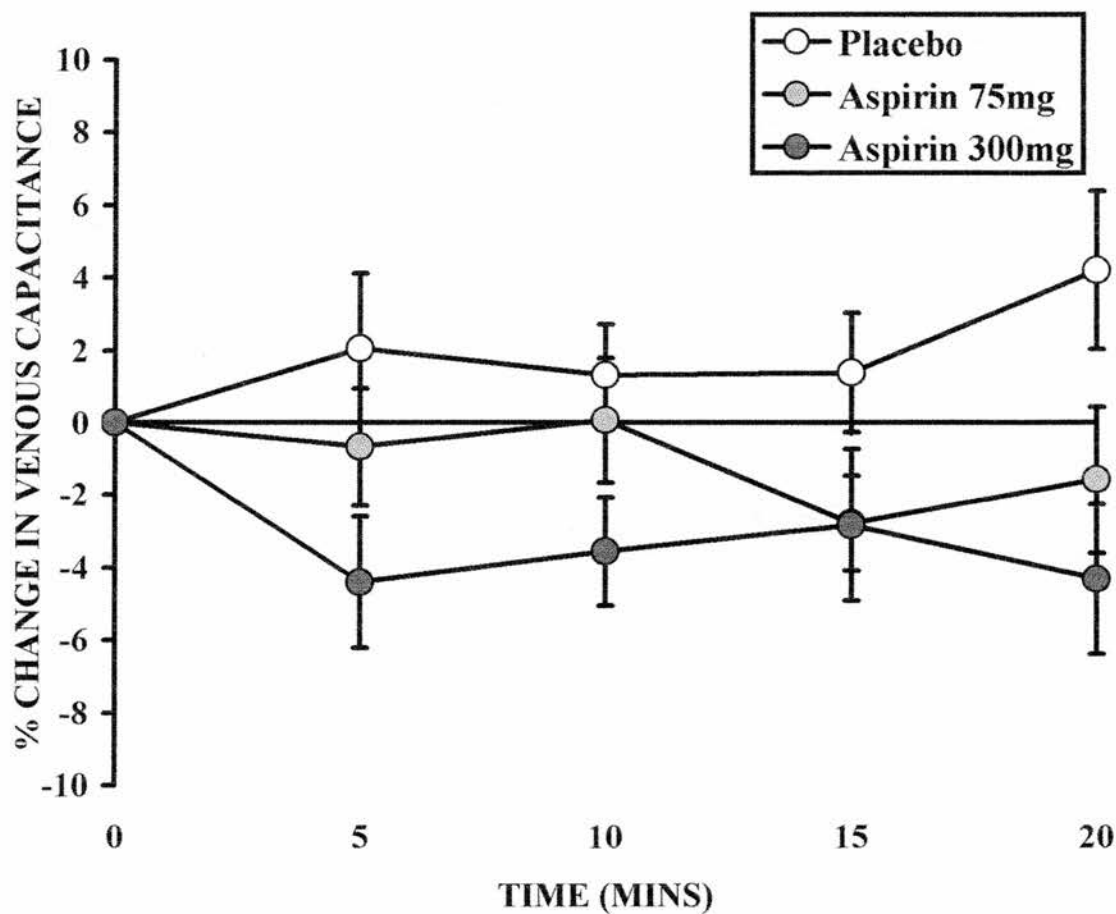
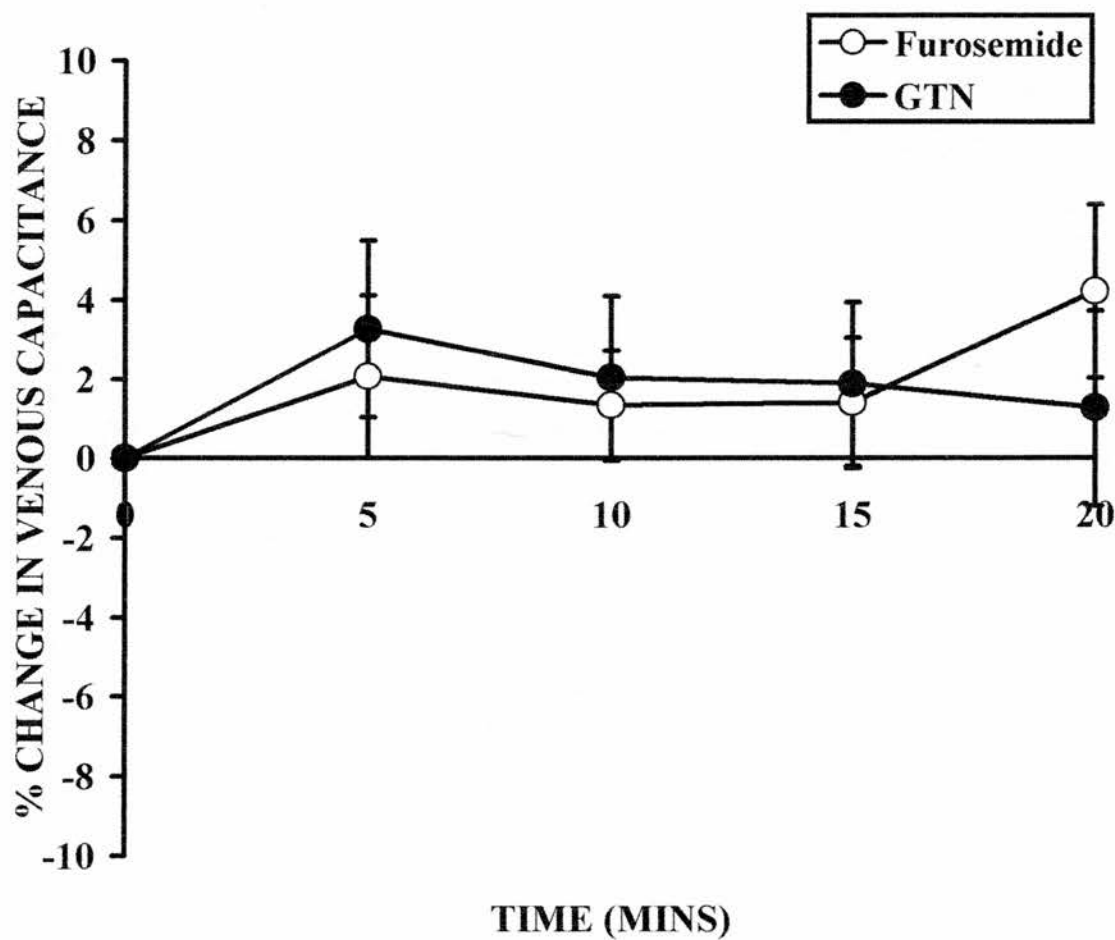


Figure 4.3 Effect of GTN on venous capacitance compared with effect of furosemide (placebo study) (p=ns)



5 Effect of bradykinin and substance P in heart failure

5.1 Introduction

As we have already observed, there is continuing controversy surrounding the concomitant use of aspirin and angiotensin converting enzyme (ACE) inhibitors in patients with congestive heart failure due to left ventricular systolic dysfunction (Cleland *et al*, 1995). Whilst this hypothesis continues to await a definitive answer, some progress can be made by studying some of the potential mechanisms for such an interaction. As previously discussed, any interaction between aspirin and ACE inhibitors must almost certainly involve prostaglandins, for while the effect of ACE inhibitors may be quite complex, those of aspirin are almost certainly quite simple (Vane *et al*, 1971). The most usual explanation for the interaction between aspirin and ACE inhibitors therefore involves bradykinin. ACE inhibitors have been shown to potentiate the effects of bradykinin by inhibition of its breakdown (Benjamin *et al*, 1989), and bradykinin has been shown to exert at least some of its effects via prostaglandins, as described in chapter 3. It is therefore an inhibition by aspirin of prostaglandin-mediated bradykinin-induced effects of ACE inhibitors which is postulated to account for their negative interaction. We therefore sought to determine whether or not aspirin actually has any effect on the response to bradykinin, in patients with heart failure treated with an ACE inhibitor. We also took advantage of the opportunity to examine the effect of aspirin on the response to another kinin, substance P. Not usually invoked as a mediator of any sort of interaction between aspirin and ACE inhibitors, substance P is a potent vasodilator, with evidence that it may be potentiated by ACE inhibitors (Valdemarsson *et al*, 1991), and that it may exert its effects via prostaglandins (Shirahase *et al*, 1995).

5.2 Methods

5.2.1 Patients

The study was conducted with the approval of the West Ethics Committee and all patients gave written informed consent. Twelve patients with chronic heart failure secondary to left ventricular systolic dysfunction confirmed by echocardiography were studied (left ventricular ejection fraction <40%). All patients were clinically stable on constant medication for 3 months, with no peripheral oedema or pulmonary congestion, and no uncontrolled hypertension, hypercholesterolaemia or diabetes mellitus. All patients were treated with an ACE inhibitor (enalapril 10mg bd in the majority). All medication apart from aspirin was constant throughout the study. Patient characteristics are summarised in Table 5.1.

5.2.2 Treatment

Each patient was studied on two occasions, after 14 days of no aspirin at all, and after 14 days of 150mg aspirin daily, having abstained from all other aspirin, aspirin-containing medications or aspirin-like medications during that time. Patients were not randomised, but studied in the order: 0mg; 150mg. This dose is in the middle of the range previously demonstrated by us to inhibit arterial vasodilation to arachidonic acid (chapter 3) and venodilation to furosemide (chapter 4). Patients took their usual medications including aspirin therapy as directed 6 hours before attendance on the day of study. Patients abstained from alcohol, tobacco and caffeine for 24 hours before each study and attended fasted for 6 hours. Bradykinin was infused at 10, 30 and 100 pmol/min for 3 minutes at each dose and substance P was infused at 1, 2 and 4 pmol/min for 3 minutes at each dose. Aspirin was then infused at

1mg/min for 15 minutes. Bradykinin and substance P were then re-infused in the same order as before (the order of infusion having been randomised). For the second study, patients attended after taking oral aspirin for 14 days, and only the first part of the study (bradykinin and substance P infusion) was repeated. Each patient therefore had measurement of responses to bradykinin and substance P off all aspirin therapy, after a single dose of intra-arterial aspirin only, and after 14 days of regular oral aspirin only (in that order). The study design is summarised in Figure 5.1.

5.2.3 Measurements

Forearm blood flow was measured by the technique of venous occlusion plethysmography as described in chapter 2. Plethysmographic recordings were made at 10 minute intervals during saline infusion and at 3-5 minute intervals during drug infusion. Blood pressure and pulse rate were manually recorded in the non-infused arm at 5-10 minute intervals throughout each study.

5.2.4 Drugs

Bradykinin (purity 99.4% by HPLC) was obtained from Clinalfa AG (Laufelfingen, Switzerland) and dissolved in normal saline. Substance P (purity 99.6% by HPLC) was obtained from Clinalfa AG and dissolved in normal saline. Aspirin was obtained from Synthelabo France and dissolved in normal saline. All drugs were always used within 2 hours of final preparation and destroyed thereafter.

5.2.5 Data analysis

All results are expressed as mean values with standard errors. All results were

compared using two-tailed paired t tests. Differences were considered significant at a value of $p < .05$.

5.3 Results

5.3.1 General effects

Local infusion of bradykinin, substance P and aspirin caused no adverse or systemic effects, and patients did not report any discomfort. Pulse rate, blood pressure, and forearm blood flow in the non-infused forearm did not change significantly during either of the studies, and there were no significant differences in baseline pulse rate, blood pressure or baseline forearm blood flow between either of the studies.

5.3.2 Effect of bradykinin

Bradykinin caused marked vasodilation, which was rapid in onset (~1 minute) and almost as rapid in offset (~5 minutes). There was obvious flushing of the whole forearm at the highest dose of bradykinin in the majority of cases, although no patient complained of this spontaneously. There was a clear dose-response relationship, with peak vasodilation at the highest dose of 100 pmol/min, and no sign of the development of significant tachyphylaxis (Figure 5.2).

5.3.3 Effect of substance P

Substance P caused vasodilation, which was even more rapid in onset and offset. The magnitude of vasodilation to substance P was rather less than that to bradykinin, at the much lower doses used. Vasodilation to 4 pmol/min of substance P

was rather more than the vasodilation to 10 pmol/min of bradykinin. There was again a clear dose-response relationship, with peak vasodilation at the highest dose of 4 pmol/min, and no sign of the development of significant tachyphylaxis (Figure 5.3).

5.3.4 Effect of intra-arterial aspirin

Intra-arterial aspirin had no significant effect on forearm blood flow on its own (Figure 5.4). There was a trend towards vasoconstriction which was significant at 15 minutes but not at 10 and 5 minutes, and not over the whole 15 minutes.

5.3.5 Effect of intra-arterial aspirin on bradykinin/substance P

Intra-arterial aspirin had no significant effect on the vasodilator response to either intra-arterial bradykinin (Figure 5.2) or substance P (Figure 5.3).

5.3.6 Effect of oral aspirin on bradykinin/substance P

Aspirin 150mg a day for 14 days had no significant effect on the vasodilator response to either intra-arterial bradykinin (Figure 5.2) or substance P (Figure 5.3). Although there appeared to be a slight reduction in the response to bradykinin, particularly at the highest dose of bradykinin, this was not significant and was in any case modest in proportion compared to the magnitude of the vasodilator response.

5.4 Discussion

In this study in the forearm of patients with heart failure, we have shown that exogenous bradykinin and substance P cause vasodilation, that intra-arterial aspirin has no obvious effect on its own, that intra-arterial aspirin has no effect on the

response to bradykinin or substance P, and that 14 days oral aspirin 150mg od has no effect on the response to bradykinin or substance P. All these findings are novel, interesting in their own right, and have wider implications.

5.4.1 Effect of bradykinin in heart failure

This is the first study we are aware of that has examined the effects of exogenous bradykinin in human patients with heart failure. Bradykinin has been studied in healthy volunteers (Fox *et al*, 1961) and in patients with endothelial dysfunction (Panza *et al*, 1995), but not previously in patients with heart failure, despite the importance of ACE inhibitors and the potential importance of bradykinin potentiation in heart failure. Our findings confirm that bradykinin is a potent vasodilator in patients with heart failure treated with an ACE inhibitor, as it is in subjects without heart failure. Furthermore, they suggest the hypothesis that this may be one endothelium-dependent response, which is not impaired in heart failure, at least not markedly. Unfortunately, we did not have the opportunity to study healthy controls, but our recordings really are remarkably similar to those seen previously in healthy volunteers also treated with an ACE inhibitor (Figure 5.5), albeit acutely rather than chronically (Cockcroft *et al*, 1993). Obviously it is possible that there is a difference between chronic and acute treatment which cancels out a difference between heart failure and normal, but interestingly it has recently been reported that responses to exogenous bradykinin are indeed unimpaired in heart failure, albeit an animal model (Su *et al*, 1998). This is perhaps all the more surprising given that heart failure has also been reported to increase plasma levels of bradykinin, also in an animal model (Cheng *et al*, 1998).

5.4.2 Effect of substance P in heart failure

Unlike bradykinin, substance P has been studied in heart failure, as well as other models of endothelial dysfunction, although the results of this have been somewhat inconsistent. The only other study to look at the effects of substance P in the forearm of patients with heart failure found that vasodilation was unimpaired, allowing for the lower basal blood flow in patients compared with controls (Hirooka *et al*, 1992). Of course, this rather begs the question of how to adjust responses for lower basal blood flow in the first place. Absolute changes in blood flow are obviously fairly meaningless, but there is no guarantee that relative changes are any more meaningful. Under these circumstances, it is perhaps no surprise that a different study examined endothelium-dependent responses in the lower limb, and found that the response to substance P was reduced in patients compared with controls, albeit to a lesser extent than the response to acetylcholine (Lindsay *et al*, 1996). Other studies have been *in vitro* (Berkenboom *et al*, 1987) or in animal models of heart failure (Rubinstein *et al*, 1994). Yet other studies have found that the response to substance P is reduced in patients with endothelial dysfunction due to hypertension (Panza *et al*, 1994) and that the response to substance P is not reduced in patients with endothelial dysfunction due to atherosclerosis (Husain *et al*, 1998). We found obvious vasodilation to substance P in patients with heart failure treated with an ACE inhibitor. There are unfortunately no previous studies of the effect of substance P in patients treated with an ACE inhibitor, and we did not have the opportunity to study the effect of substance P in healthy controls. Although comparison with the effects of bradykinin suggests this vasodilation is quite modest, the molar doses of substance P were multiple orders of magnitude smaller. The role of substance P as a potent

vasodilator, even in heart failure, at least when treated with an ACE inhibitor, is hereby reiterated.

5.4.3 Effect of intra-arterial aspirin

At first site, our findings with intra-arterial aspirin would appear to be markedly at odds with the one previous study to report directly on the effects of intra-arterial aspirin. Duffy *et al* (1998) found a significant reduction in forearm blood flow with intra-arterial aspirin infusion. However, the two sets of findings are not as incompatible as they might at first sight appear to be. Comparing the time course of our findings at 1 mg/min with the previous findings at 3 mg/min reveals only one time point (10 minutes), when our findings were significantly different (Figure 5.6). Comparing our findings with the dose-response curve of the previous study reveals that our findings at 1 mg/min were compatible with the previous findings at the same dose (Figure 5.7). We deliberately chose to avoid higher doses or a more prolonged infusion because of the risk that that would lead to supra-pharmacoactive concentrations in the forearm, or even pharmacoactive concentrations systemically. Of course the slight difference between our findings and the previous findings could relate to a difference between health and disease or a difference between treatment and no treatment.

5.4.4 Interaction of bradykinin and aspirin

Our findings show no significant effect of aspirin, whether intra-arterial or oral, on the vasodilator response to bradykinin in patients with heart failure treated with an ACE inhibitor. The effects of bradykinin are frequently said to be aspirin-

sensitive and this sensitivity is frequently invoked as a potential mechanism for the reported negative interaction between aspirin and ACE inhibitors. Despite this, the evidence that aspirin actually affects the vascular effects of bradykinin is patchy (see also discussion in chapter 3). There is no doubt that aspirin blocks the algescic effects of bradykinin (Coffman *et al*, 1966), indeed this is probably central to the analgesic activity of aspirin and aspirin-like drugs (Moncada *et al*, 1975). There are also well-validated instances of inhibition of smooth muscle motor responses to bradykinin by aspirin (Ono *et al*, 1977; Walker *et al*, 1979), and yet the effect of aspirin on vasomotor responses to bradykinin is much more variable. There seems no doubt that aspirin blocks the release of vasoactive prostanoids by bradykinin (Heavey *et al*, 1985; Ritter *et al*, 1989; Clarke *et al*, 1991; Keimowitz *et al*, 1993). Despite this there are *at least* four reports (Benjamin *et al*, 1989; Heavey *et al*, 1985; Ritter *et al*, 1989; Bhagat *et al*, 1995) that aspirin has no effect on the vasodilator response to bradykinin. Most of the reports that aspirin does block the vasomotor effects of bradykinin have been *in vitro* (Toda *et al*, 1977; Fasciolo *et al*, 1990) or in animals (Fasciolo *et al*, 1990; Turker *et al*, 1982; Copeland *et al*, 1995). Our findings add to the evidence that aspirin does not block the vasodilator effect of bradykinin, and extend that finding to patients with heart failure treated with an ACE inhibitor, a group in whom this is of considerable interest.

5.4.5 Interaction of substance P and aspirin

Our findings show no significant effect of aspirin, whether intra-arterial or oral, on the vasodilator response to substance P in patients with heart failure treated with an ACE inhibitor. Potentiation of substance P by inhibition of its breakdown is

frequently invoked as a possible cause of side effects such as cough in patients treated with an ACE inhibitor (Emanuelli *et al*, 1998; Yeo *et al*, 1995). This is despite the fact that the most direct examination of the interaction between substance P and an ACE inhibitor found absolutely no evidence of any effect whatsoever (Benjamin *et al*, 1990). Any evidence that the effects of substance P were mediated by prostaglandins and blocked by aspirin might still be highly pertinent to the reported negative interaction between aspirin and ACE inhibitors. As with bradykinin, there is little doubt that aspirin blocks the algescic effects of substance P (Hunnskaar *et al*, 1985), and again, this is perhaps somewhat central to the analgesic effect of aspirin. As with bradykinin, the evidence that aspirin blocks the vasodilator effects of substance P is patchy, venodilation apparently being blocked (Strobel *et al*, 1996), and arterial vasodilation (at least in patients with atherosclerosis) apparently not (Husain *et al*, 1998). Our findings add to the evidence that aspirin does not block the vasodilator effect of substance P, at least in patients with heart failure treated with an ACE inhibitor. This is a group in whom this is of considerable interest, given that substance P levels are elevated in heart failure (Edvinsson *et al*, 1990), and further elevated by treatment with ACE inhibitors (Valdemarsson *et al*, 1991). We can only assume that the mechanism for this elevation, if it is real, is an indirect rather than a direct one.

5.4.6 Summary

We have shown that exogenous bradykinin and substance P cause vasodilation in patients with heart failure, that there is no evidence of any effect of intra-arterial aspirin on its own, and that there is no evidence of any effect of aspirin, intra-arterial or oral, on the responses to bradykinin and substance P. Given the fairly small

numbers involved, we cannot rule out a type II error (i.e., a false negative), but the strength of the response to bradykinin and substance P and the lack of any obvious response to aspirin rules out anything but quite a subtle influence of aspirin on these mediators. Demonstration of these responses is completely novel in patients with heart failure, and is only partly consistent with previous work *in vitro*, in animals and in healthy volunteers. Whilst these findings leave open the issue of whether bradykinin or substance P contributes to the non-angiotensinergic effects of ACE inhibitors, they do suggest that neither bradykinin nor substance P can be invoked as a mechanism to explain the reported negative interaction between aspirin and ACE inhibitors, and proponents of this hypothesis will need to look to other mediators to incriminate, perhaps, for example, angiotensin-(1-7) (Ferrario *et al*, 1991).

Table 5.1 Patient Characteristics

<i>Patient Characteristic</i>	
Sex, n	10 M/2 F
Age, y (mean±SD)	63±6
Primary diagnosis IHD/IDC, n	10/2
NYHA functional class II/III/IV, n	7/4/1
LVEF, % (mean±SD)	20±7
Current smoker, n	4
On Diuretic, n	12
ACE inhibitor, n	12
Enalapril 10mg bd, n	9
Aspirin, n	9
Statin, n	6
Beta blocker, n	3
Digoxin, n	3
Nitrate, n	1

IHD = ischaemic heart disease; IDC = idiopathic dilated cardiomyopathy; NYHA = New York Heart Association; LVEF = left ventricular ejection fraction

Figure 5.1 Study design

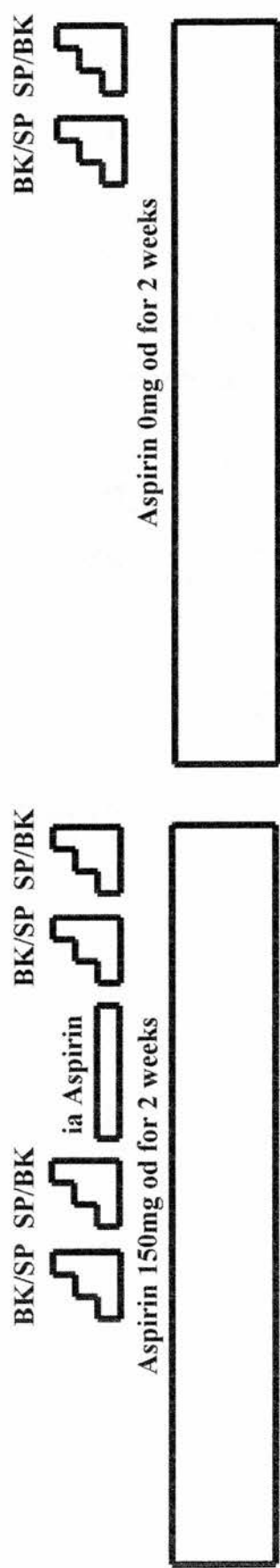


Figure 5.2 Effect of bradykinin on forearm blood flow before and after intra-arterial and oral aspirin (BK = no aspirin; BK+A(IA) = after intra-arterial aspirin; BK+A(O) = after 14 days oral aspirin: p=ns for BK versus BK+A(IA), BK+A(IA) versus BK+A(O) and BK+A(O) versus BK)

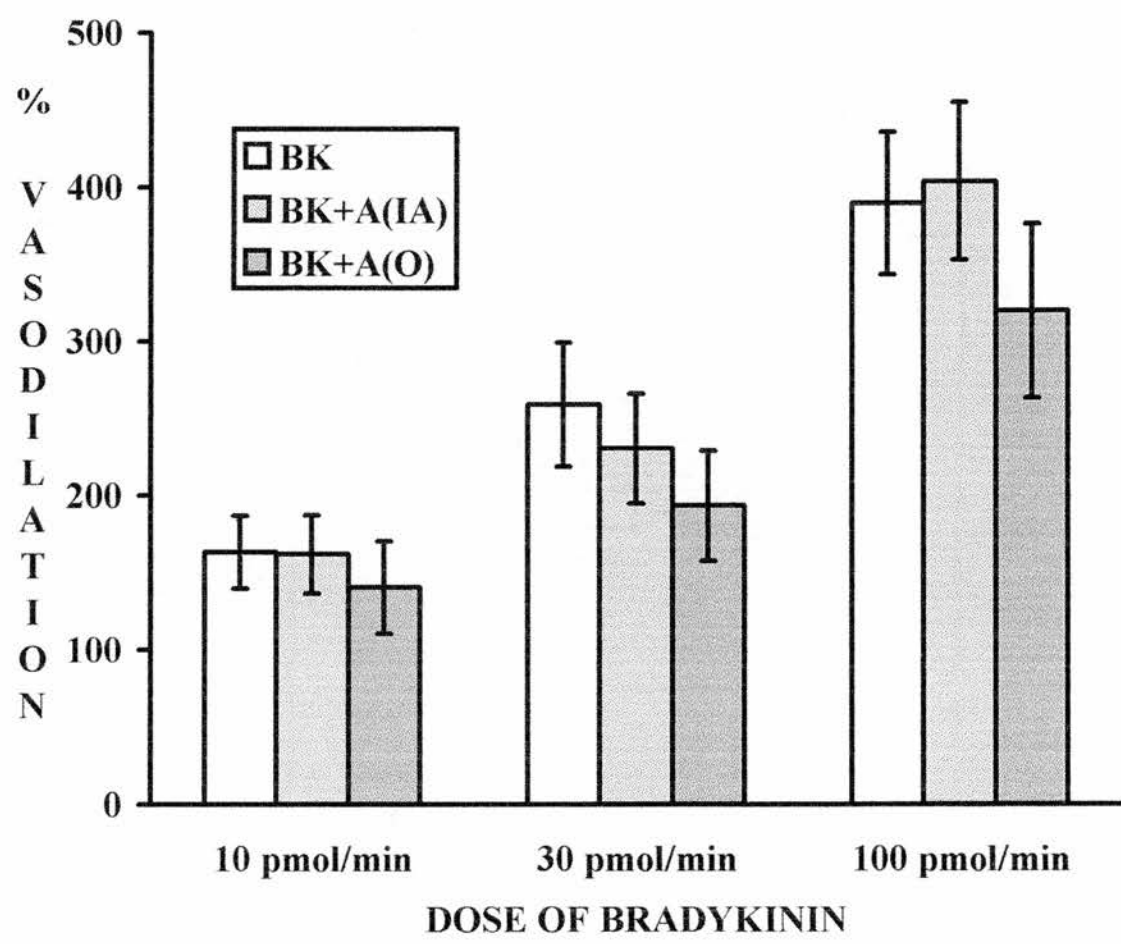


Figure 5.3 Effect of substance P on forearm blood flow before and after intra-arterial and oral aspirin (SP = no aspirin; SP+A(IA) = after intra-arterial aspirin; SP+A(O) = after 14 days oral aspirin: p=ns for SP versus SP+A(IA), SP+A(IA) versus SP+A(O) and SP+A(O) versus SP)

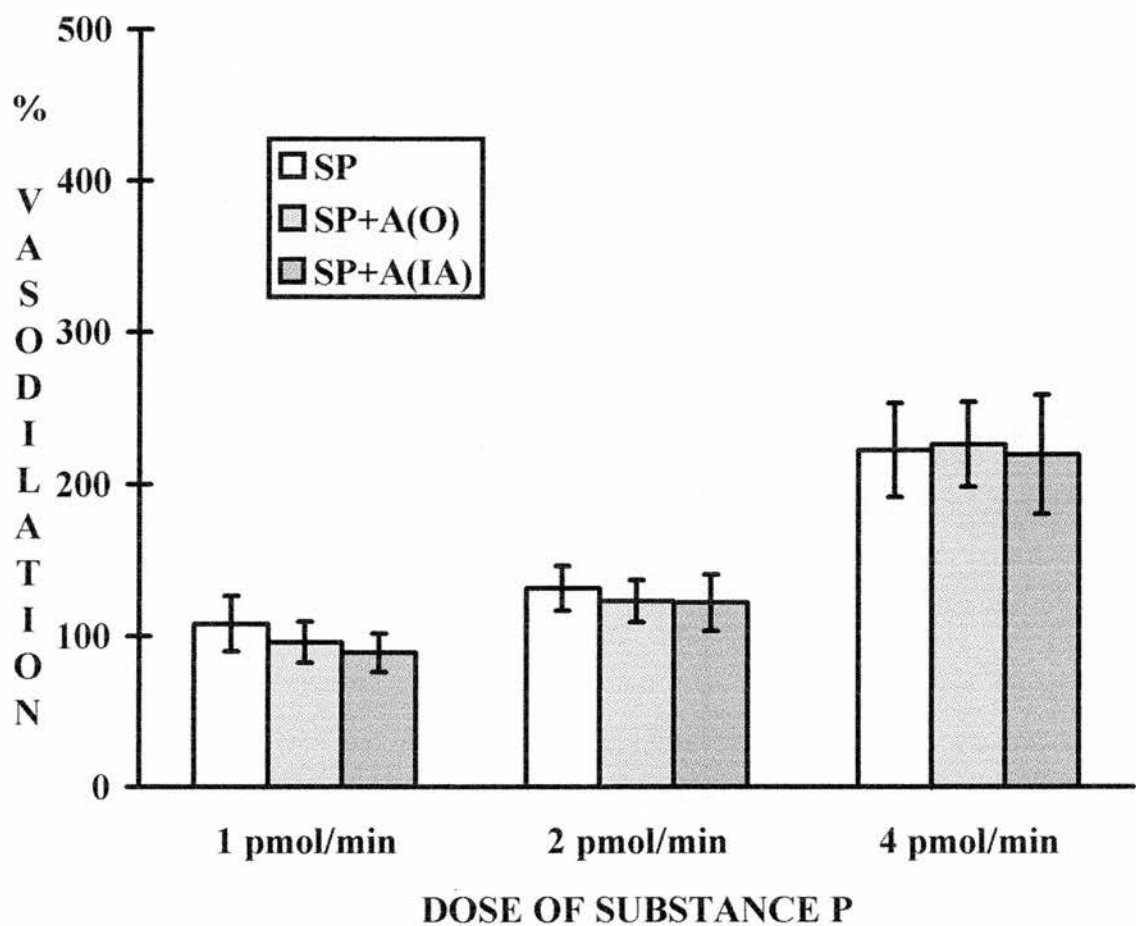


Figure 5.4 Effect of intra-arterial aspirin on forearm blood flow

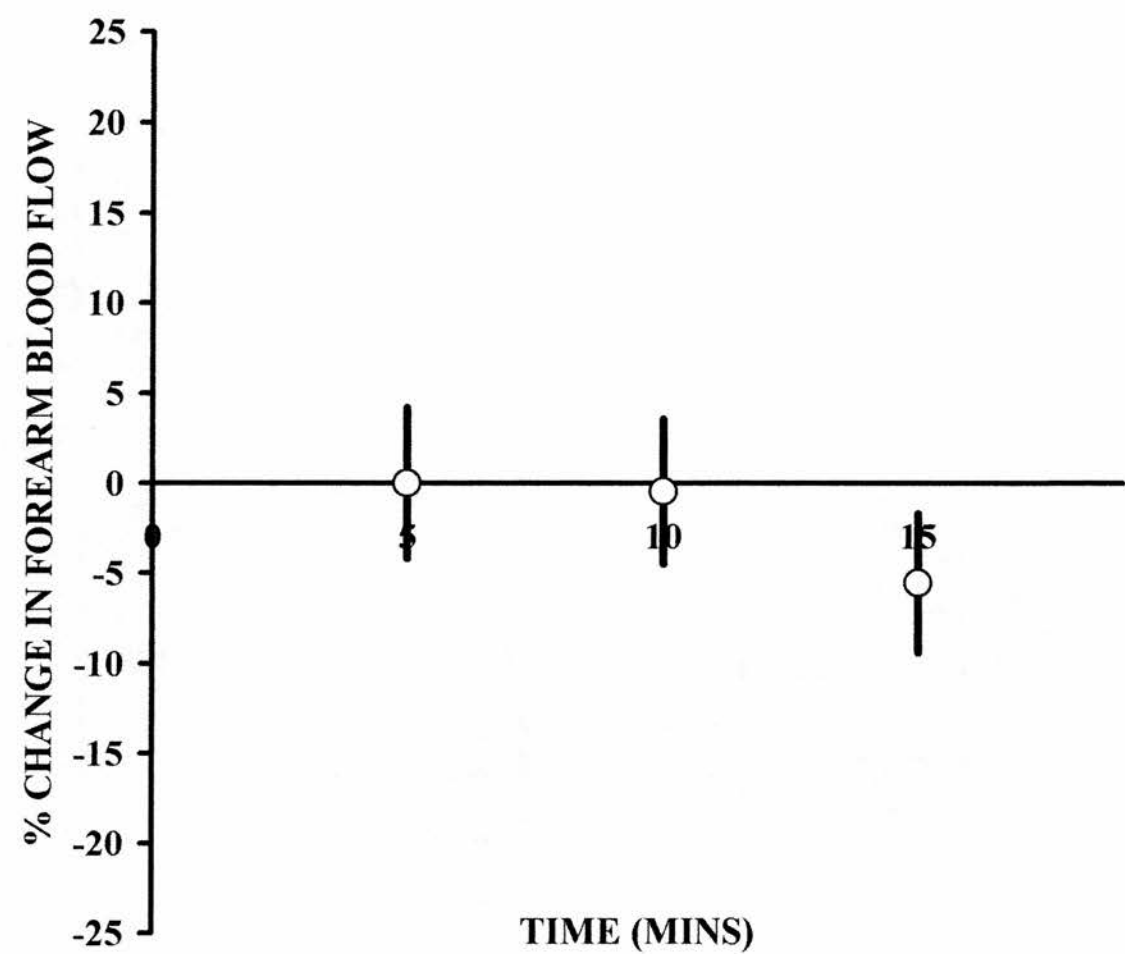


Figure 5.5 Comparison between our results with bradykinin (in heart failure patients established on ACE inhibitor) & those of Cockcroft et al (in healthy volunteers studied after a single dose of enalapril 10mg)

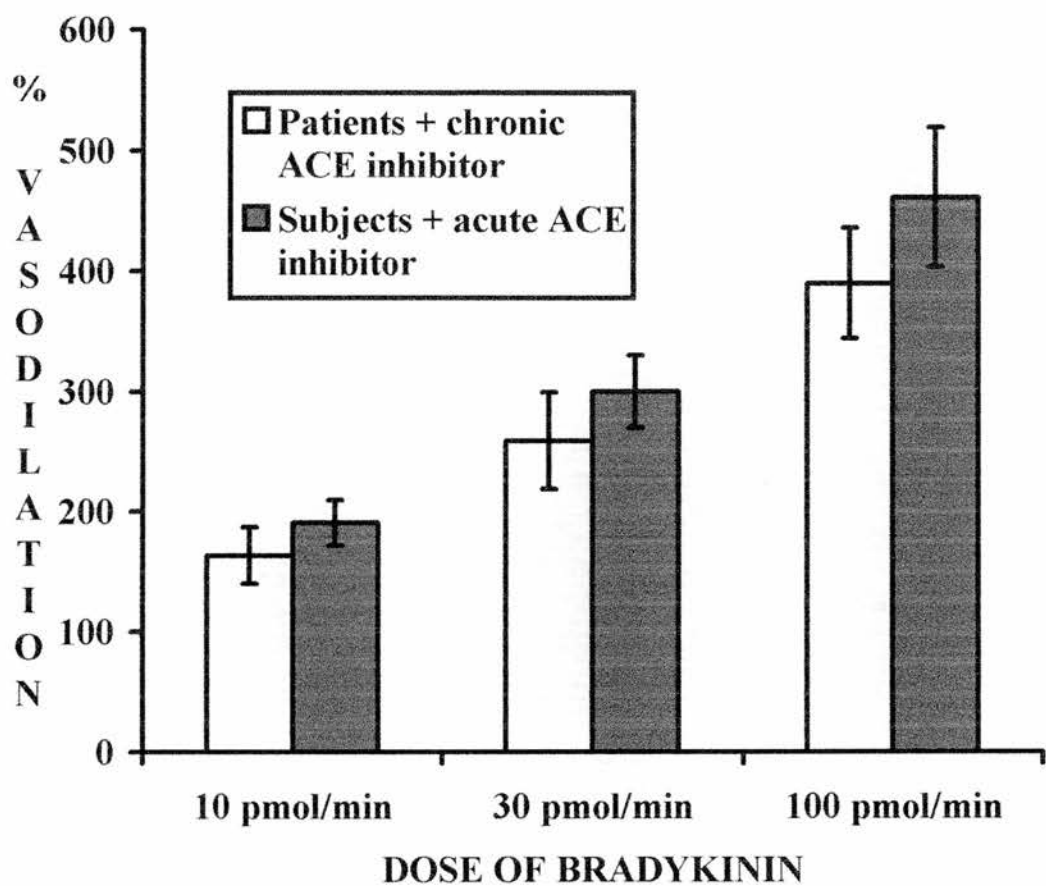


Figure 5.6 Comparison between the time course of our results with aspirin (1 mg/min) and those of Duffy et al (3 mg/min)

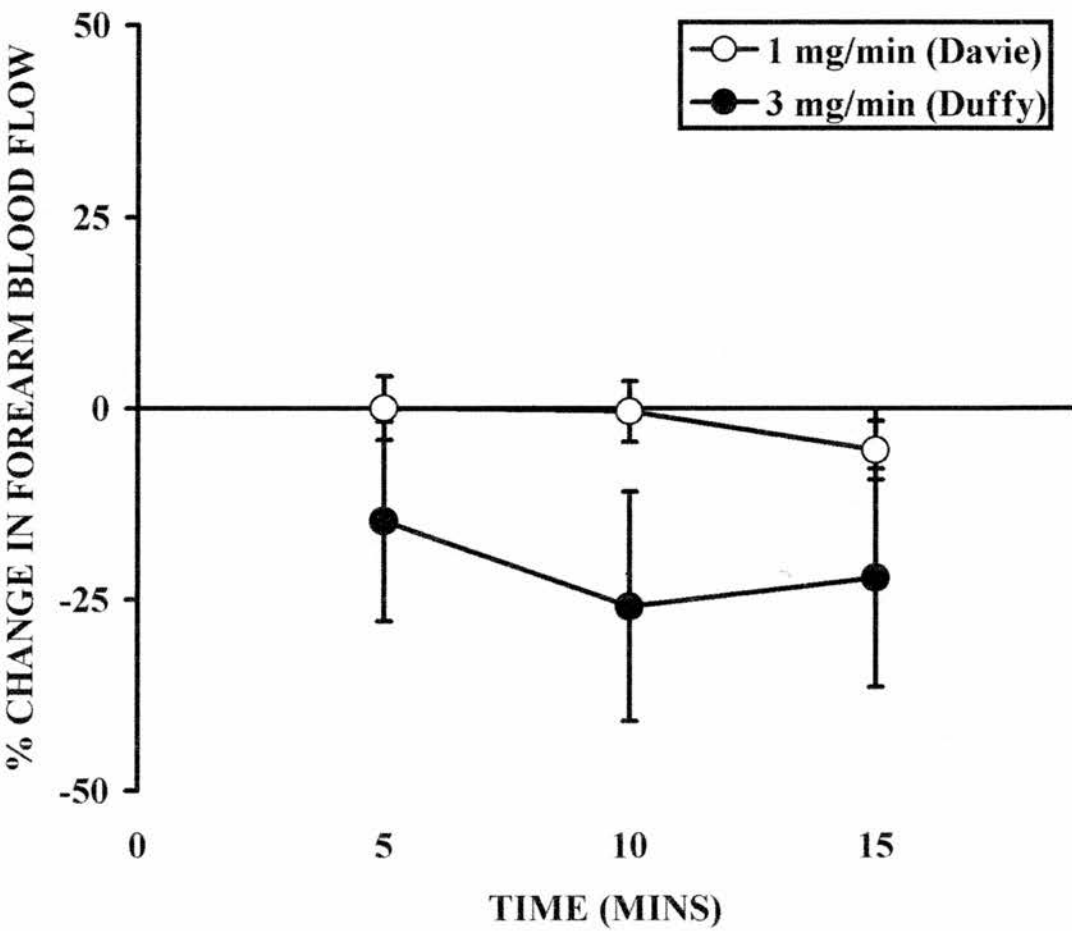
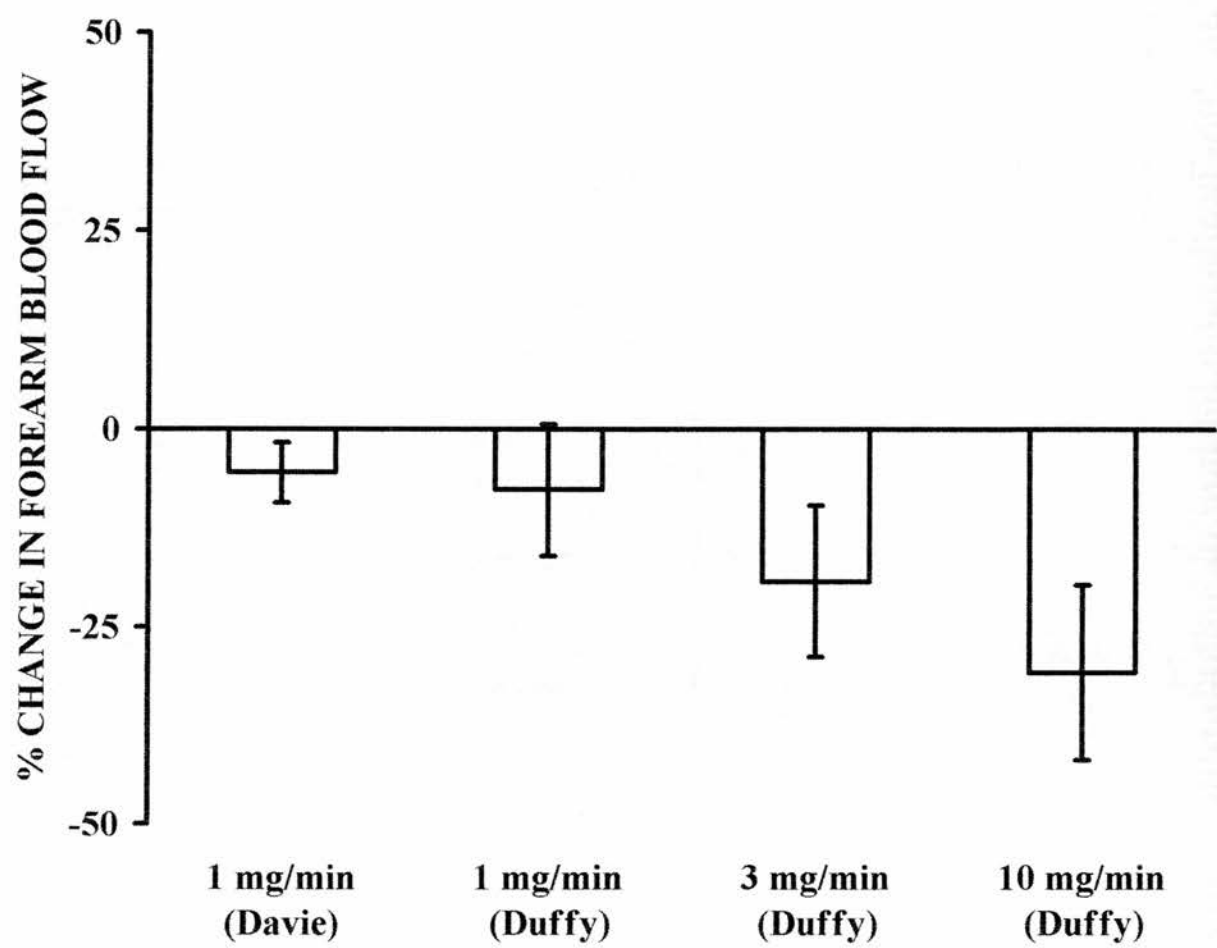


Figure 5.7 Comparison between dose-response of our results with aspirin (1 mg/min) and those of Duffy et al (1 - 3 - 10 mg/min)



6 Effect of bradykinin antagonism in heart failure

6.1 Introduction

It has long been known that angiotensin converting enzyme (ACE) is identical to kininase II, the most important pathway for degradation of bradykinin (Yang *et al*, 1970). Indeed, when parenterally active ACE inhibitors first became available they were known as bradykinin-potentiating peptides (Gavras *et al*, 1974). Parenterally active angiotensin II type I receptor (AT1) antagonists became available around the same time (Solomon *et al*, 1974). Shortly afterwards, orally active ACE inhibitors became available (Gavras *et al*, 1978), and the focus of interest switched to their effects alone, as it was a long time before orally active AT1 antagonists became available (Chiu *et al*, 1990). Now that orally active ACE inhibitors and AT1 antagonists are both readily available, the focus of interest is switching from their similarities towards their differences. The most obvious difference remains that ACE inhibitors are bradykinin-potentiating whereas AT1 antagonists are not, at least, not directly, as far as we know. Despite this, it is not known to what extent endogenous bradykinin-potential actually contributes to the beneficial effects or side effects of ACE inhibitors in patients with heart failure. Whilst it is indisputable that AT1 antagonists have a better side effect profile than ACE inhibitors (Pitt *et al*, 1997), it is not known at all whether they share the same beneficial effects overall. As work continues to develop the use of AT1 antagonists in heart failure, it is important that their differences from ACE inhibitors are scrutinised, and the role of bradykinin in the effects of either or both better defined. Recently, a potent, selective and long-acting bradykinin type 2 receptor (B2) antagonist, Hoe-140 (for which the name icatibant has also been proposed), has become available (Hock *et al*, 1991; Wirth *et al*, 1991). This gives us an exciting opportunity to scrutinise the effects of bradykinin in this

area, as most of the clinically relevant effects of bradykinin are mediated through the B2 receptor (Regoli *et al*, 1980, 1990). In this study, we examined the effects of bradykinin, Hoe-140, and their combination in patients with heart failure randomised to double-blind cross-over treatment with an ACE inhibitor and an AT1 antagonist.

6.2 Methods

6.2.1 Patients

The study was conducted with the approval of the West Ethics Committee and all patients gave written informed consent. Twelve patients with chronic heart failure secondary to left ventricular systolic dysfunction confirmed by echocardiography were studied (left ventricular ejection fraction <40%). All patients were clinically stable on constant medication for 3 months, with no peripheral oedema or pulmonary congestion, and no uncontrolled hypertension, hypercholesterolaemia or diabetes mellitus. All patients were treated with an ACE inhibitor at baseline (enalapril 10mg bd in the majority). All medication apart from ACE inhibitor/AT1 antagonist and aspirin was constant throughout the study. Patient characteristics are summarised in Table 6.1. Eight of the twelve patients also took part in a pilot and dose-ranging study whilst taking their usual ACE inhibitor, in order to confirm the safety of intrabrachial Hoe-140 and establish an appropriate dose for demonstration of its arterial vasomotor effects (if any).

6.2.2 Randomisation

Patients were randomised to double-blind cross-over treatment with enalapril 10mg bd for 5 weeks followed immediately by losartan 25mg bd for 5 weeks, or *vice*

versa. The doses of enalapril and losartan were both chosen on the basis of the best-available evidence, randomised controlled trials with proper clinical endpoints. The dose of enalapril was taken from the SOLVD trials (1991; 1992), and the dose of losartan was taken from the ELITE study (Pitt *et al*, 1997), split into two doses for ease of blinding. Although we cannot be certain that these doses are equivalent, it is so difficult to settle on equivalent doses of different ACE inhibitors, let alone ACE inhibitors and other drugs, that randomised controlled trials with proper clinical endpoints are all we have to go on. At the end of each 5 week study period patients attended for venous occlusion plethysmography, having abstained from all aspirin therapy for 14 days. Patients took their usual medications except for aspirin and including enalapril/losartan 6 hours before attendance on the day of study. Patients abstained from alcohol, tobacco and caffeine for 24 hours before each study and attended fasted for 6 hours.

6.2.3 Treatment

In the first few pilot studies, Hoe-140 was infused at 90 $\mu\text{g}/\text{min}$ for 15 minutes, as alluded to in the study by Groves *et al* (1995). When further dose-ranging studies were required, the doses arbitrarily chosen were 45, 23, 12 and 6 $\mu\text{g}/\text{min}$ each for 15 minutes. The dose finally settled on was 7 $\mu\text{g}/\text{min}$ for 30 minutes (the total dose was therefore 210 μg).

In the main study, bradykinin was infused at 10, 30 and 100 pmol/min for 3 minutes at each dose, Hoe-140 was infused as above, and then bradykinin was re-infused as before. After the first study patients immediately crossed-over to the other

double-blind treatment period, at the end of which they returned for the second study which was conducted in exactly the same way. The study protocol is summarised in Figure 6.1.

6.2.4 Measurements

Forearm blood flow was measured by the technique of venous occlusion plethysmography as described in chapter 2. Plethysmographic recordings were made for a period of 2½ minutes at 10 minute intervals during saline infusion and at 3-5 minute intervals during drug infusion. Blood pressure and pulse rate were manually recorded in the non-infused arm at 5-10 minute intervals throughout the study.

6.2.5 Drugs

Bradykinin (purity 99.4% by HPLC) was obtained from Clinalfa AG (Laufelfingen, Switzerland) and dissolved in normal saline. Hoe-140 (purity 99.9% by HPLC) was obtained from Peptides International (Louisville, Kentucky, USA) and dissolved in normal saline. Both drugs were always used within 2 hours of final preparation and destroyed thereafter.

6.2.6 Data analysis

All results are expressed as mean values with standard errors. All results were compared using two-tailed paired *t* tests. Differences were considered significant at a value of $p < .05$.

6.3 Results

6.3.1 General effects

Local infusion of bradykinin and Hoe-140 caused no adverse or systemic effects, and patients did not report any discomfort. Pulse rate (Figure 6.2), blood pressure (Figure 6.3), and forearm blood flow in the non-infused forearm did not change significantly during either of the studies. There were no significant differences in baseline pulse rate (Figure 6.2), blood pressure (Figure 6.3) or baseline forearm blood flow between either of the studies (although there was a trend towards a lower blood pressure and a higher baseline forearm blood flow in the enalapril study compared with the losartan study).

6.3.2 Pilot and dose-ranging studies

The initial four studies were closely modelled after the pilot study alluded to by Groves *et al* (1995), as this was the only mention of any forearm studies with Hoe-140 in the literature at the time. Hoe-140 was therefore infused at 90 $\mu\text{g}/\text{min}$, albeit for 20 minutes rather than the 15 minutes of the previous studies. There was no obvious effect of this dose on its own, but it did result in profound blockade of the vasodilator effects of exogenous bradykinin. This was in marked contrast to the result of Groves *et al*, who reported that the same dose caused a 24% reduction in forearm blood flow (this was a pilot study for the main study of the effects of Hoe-140 on coronary blood flow). The only way we could explain this (apart from Hoe-140 having no effect) was to postulate that perhaps Hoe-140 was acting as a partial agonist, i.e., a mixed antagonist and agonist. We therefore set about finding the minimum dose which provided effective blockade of the effects of exogenous

bradykinin (arbitrarily considered to be 50% inhibition of the effects of the highest dose of bradykinin used). The doses tried were 45, 23, 12 and 6 $\mu\text{g}/\text{min}$ each for 15 minutes, and whilst there were no obvious effects of any of these on their own, all provided varying degrees of blockade of exogenous bradykinin. We therefore settled on a 30 minute infusion of 7 $\mu\text{g}/\text{min}$ as a compromise.

6.3.3 Effect of bradykinin

Bradykinin caused marked vasodilation which was rapid in onset (~ 1 minute) and almost as rapid in offset (~ 5 minutes). There was obvious flushing of the whole forearm at the highest dose of bradykinin in the majority of cases, although no patient complained of this spontaneously. There was a clear dose-response relationship, with peak vasodilation at the highest dose of 100 pmol/min , and no sign of the development of significant tachyphylaxis (Figure 6.4). There was a very obvious effect of pre-treatment, with more vasodilation to bradykinin in the enalapril study than in the losartan study ($163 \pm 33\%$; $248 \pm 62\%$; $357 \pm 67\%$ versus $36 \pm 10\%$; $103 \pm 19\%$; $230 \pm 46\%$; $p=0.0002$). Indeed, in the middle of the dose-response range demonstrated, vasodilation to 100 pmol/min of bradykinin in the losartan study was not significantly different from that to 30 pmol/min or 10 pmol/min in the enalapril study ($230 \pm 46\%$ versus $248 \pm 62\%$, $p=\text{ns}$; $230 \pm 46\%$ versus $163 \pm 33\%$, $p=\text{ns}$).

6.3.4 Effect of Hoe-140

Hoe-140 had no effect on its own, either in the enalapril study or in the losartan study. Although there appeared to be slight vasoconstriction to Hoe-140 in the enalapril study, this was not significantly different from the response in the

losartan study, and only slightly different from baseline. Even if this were taken to signify vasoconstriction, which our study is underpowered to detect, it is extremely mild in degree (vasoconstriction at 30 minutes, $4\pm2\%$, Figure 6.5). Furthermore, a dose of Hoe-140 almost seven times higher, given to four out of the twelve patients whilst taking their usual ACE inhibitor, produced a similar lack of response (an apparent vasodilation of $8\pm2\%$).

6.3.5 Effect of bradykinin after Hoe-140

Bradykinin again caused vasodilation, rather less than before, although the dose-response relationship was clearly preserved (Figure 6.4). This was certainly not due to the development of tachyphylaxis. At the lower doses of bradykinin, vasodilation was almost completely abolished. The effect of pre-treatment with enalapril or losartan was again clearly preserved. In the middle of the response-range demonstrated, vasodilation to bradykinin following Hoe-140 in the enalapril study was not significantly different from that preceding Hoe-140 in the losartan study (peak vasodilation $192\pm46\%$ versus $230\pm46\%$, $p=\text{ns}$). Furthermore, vasodilation to 100 pmol/min of bradykinin following Hoe-140 was not significantly different from that to 30 pmol/min or 10 pmol/min preceding Hoe-140 in the enalapril study ($192\pm26\%$ versus $248\pm62\%$, $p=\text{ns}$; $192\pm26\%$ versus $163\pm33\%$, $p=\text{ns}$). Vasodilation to bradykinin following Hoe-140 in the losartan study was very modest indeed compared to the other responses in this study (peak vasodilation $66\pm13\%$).

6.4 Discussion

In this study we have shown that exogenous bradykinin causes vasodilation in the forearm of patients with heart failure, that this response is enhanced by pre-treatment with an ACE inhibitor as compared with an AT1 antagonist, and that endogenous bradykinin antagonism with Hoe-140 has no significant effects on its own, irrespective of pre-treatment with an ACE inhibitor or an AT1 antagonist, even though it provides effective antagonism of exogenous bradykinin. All these findings are novel, interesting in their own right, and have wider implications.

6.4.1 Effect of bradykinin in heart failure

Our findings with bradykinin in this study replicate and validate our findings with bradykinin in chapter 5. The findings from this study confirm that bradykinin is a potent vasodilator in patients with heart failure, as it is in subjects without heart failure. As we have already observed it has recently been reported that responses to bradykinin are unimpaired in an animal model of heart failure (Su *et al*, 1998), but at the time of writing ours are the only studies of the effect of bradykinin in humans with heart failure in the entire literature (Davie *et al*, 1999).

6.4.2 Bradykinin and ACE inhibitors and AT1 antagonists

It is known that ACE inhibition increases forearm blood flow in response to bradykinin (Benjamin *et al*, 1989). Indeed, one previous study has compared the effect of enalapril with that of losartan on the forearm blood flow response to bradykinin (Cockcroft *et al*, 1993). Our study usefully adds to what is known about the role of bradykinin in the effects of ACE inhibitors and AT1 antagonists. Rather

than healthy volunteers, our study used patients with heart failure, the very patients in whom the effects of ACE inhibitors and AT1 antagonists, and the role of bradykinin, are of most concern. Also, we examined the effects of chronic (5 weeks) oral treatment with enalapril/losartan, rather than the acute response examined after intra-arterial enalaprilat (Benjamin *et al*, 1989), or a single oral dose of enalapril/losartan (Cockcroft *et al*, 1993). It is well known that the acute effects of ACE inhibitors may differ quite markedly from their chronic effects (Sharpe *et al*, 1980; Juillerat *et al*, 1990; Drexler *et al*, 1989), which are obviously of more clinical interest. It is not known to what extent the chronic effects of AT1 antagonists might differ from their acute effects. Despite these considerations, our findings, in patients with heart failure studied after chronic treatment, really are *remarkably* similar to those in healthy volunteers studied after acute treatment (Figure 6.6) (Cockcroft *et al*, 1993). This is perhaps even more surprising given that ACE inhibitors have been reported to cause down-regulation of bradykinin receptors (Yasujima *et al*, 1981). Obviously it is possible that some sort of difference between patients and volunteers is cancelled out by chronic versus acute treatment, or *vice versa*. In any case, our findings confirm what has been found previously, and extend those findings to a more clinically relevant setting.

6.4.3 Effect of bradykinin antagonism

It is surprising that we found no significant effect of antagonism of endogenous bradykinin with Hoe-140. It has been reported that intra-arterial Hoe-140 on its own has no significant effect on resting blood flow, although it reduces the increase in radial artery diameter (but not the increase in blood flow) following wrist

arterial occlusion (Hornig *et al*, 1997). The only other published report of the effects of Hoe-140 in the forearm of humans showed no haemodynamic effect of intravenous administration (Cockcroft *et al*, 1994). These previous studies were in healthy volunteers, rather than patients with heart failure on any sort of treatment. Even in patients with heart failure, even when treated with an ACE inhibitor, we found no acute haemodynamic effect of antagonism of endogenous bradykinin with Hoe-140. This suggests that there is a complete discrepancy between the very obvious potentiation of exogenous bradykinin by ACE inhibitors and the lack of evidence of potentiation of endogenous bradykinin. These findings are of course consistent with the evidence that ACE inhibitors do not actually increase plasma levels of bradykinin (Johnston *et al*, 1979; Johnston *et al*, 1981).

6.4.4 Interaction of bradykinin antagonism and ACE inhibition

Our findings would initially appear to be at odds with a recent report that some (but not all) of the acute haemodynamic effects of oral captopril are attenuated by co-administration by of intravenous Hoe-140 (Gainer *et al*, 1998). This implies that potentiation of endogenous bradykinin *does* contribute to the haemodynamic effects of ACE inhibition. Apart from the superficial differences between this study and our own (hypertensives versus heart failure patients, captopril versus enalapril, intravenous versus intra-arterial Hoe-140), the most important difference is that of timing. The doses of Hoe-140 were certainly of the same order of magnitude. Whilst we administered 210µg over a period of 30 minutes in the forearm (average mass ~1kg), Gainer *et al* administered a bolus of 100 µg/kg. Whereas Gainer *et al* examined the effect of Hoe-140 on the effects of a single dose of captopril, we

examined the effect of Hoe-140 in patients treated with enalapril (or losartan) for 5 weeks. As we have already observed, the chronic effects of ACE inhibitors may be quite different from their acute effects (Sharpe *et al*, 1980; Juillerat *et al*, 1990; Drexler *et al*, 1989). Gainer *et al* made a similar observation, and called for a study of the role of bradykinin during long term ACE inhibition. Our findings answer that call.

6.4.5. Local versus systemic effects

One further difference between Gainer *et al*'s study and our own is that between a systemic haemodynamic study and a local haemodynamic study. Obviously each has its strengths and weaknesses and probably both are required for full characterisation of any mechanism. The discrepancy might be construed as evidence that forearm vascular responses are not relevant to the effect of ACE inhibitors. Apart from the fact that enalaprilat has been shown to be directly vasodilating in the forearm (Bank *et al*, 1991), as have angiotensin receptor blockers (Newby *et al*, 1997), it is well-established that ACE inhibitors do increase forearm blood flow chronically if not necessarily acutely (Faxon *et al*, 1982). Furthermore, even when intrabrachial enalaprilat has failed to have any effect on its own, it has clearly reduced responsiveness to intrabrachial angiotensin I and increased responsiveness to intrabrachial bradykinin (Benjamin *et al*, 1989), just as enalapril has affected the latter in this study.

6.4.6 Effectiveness of bradykinin antagonism

The final part of our study confirmed that intra-arterial Hoe-140 antagonised

the effects of exogenous bradykinin, despite the lack of any effect of Hoe-140 on its own. It has previously been shown that intravenous Hoe-140 antagonises the effects of intra-arterial bradykinin (Cockcroft *et al*, 1994), and it has previously been shown that intra-arterial Hoe-140 blocks the effect of intra-arterial quinaprilat on flow-dependent dilation in the form of reactive hyperaemia (Hornig *et al*, 1997). This is the first study of the interaction of intra-arterial Hoe-140 and intra-arterial bradykinin, and the interaction of that interaction with chronic oral (5 weeks) treatment with ACE inhibitor/AT1 antagonist, in patients with or without heart failure. The dose of Hoe-140 used was lower than previous studies (Hornig *et al*, 1997), because we deliberately sought to use the lowest dose capable of providing effective antagonism of exogenous bradykinin. A higher dose of Hoe-140 had a similar lack of obvious effect on its own. It is clear that the lower dose did not provide complete antagonism of exogenous bradykinin, although it did do so at the lower doses of bradykinin used. It is important to note that the doses of bradykinin used were massively supra-physiological, perhaps by as much as three orders of magnitude (Hornig *et al*, 1997), and the level of blockade achieved must therefore be regarded as similarly profound. The possibility that the reduction observed could be ascribed to tachyphylaxis can be discounted, as our own observations (chapters 4 & 6) and others' (Cheng *et al*, 1998), have shown that although tachyphylaxis to bradykinin occurs, it is much more modest.

6.4.7 Interaction between aspirin and ACE inhibitors

Our findings are also relevant to the ongoing controversy surrounding the potential negative interaction between ACE inhibitors and aspirin (Hall *et al*, 1992). If such an interaction exists it is usually postulated that it is because of the effect of

aspirin on prostanoid-mediated effects of bradykinin (Cleland *et al*, 1995). It now seems possible that exogenous bradykinin potentiation may turn out to be an epiphenomenon of ACE inhibition, not necessarily relevant to the effects of ACE inhibition on endogenous bradykinin activity. In this case, there will then be no obvious substrate on which aspirin can exert its effects, and the proponents of this theory will have to look elsewhere for evidence to sustain their hypothesis.

6.4.8. Summary

We have shown that exogenous bradykinin does cause vasodilation in patients with heart failure and that this response is markedly enhanced by pre-treatment with an ACE inhibitor as compared with an AT1 antagonist. This is despite the fact that there is no evidence of an effect of endogenous bradykinin antagonism with Hoe-140, irrespective of pre-treatment with an ACE inhibitor or an AT1 antagonist. Simultaneous demonstration of this discrepancy is completely novel and has never before been achieved in patients with heart failure, although it is largely consistent with previous work *in vitro*, in animals and in healthy volunteers. This leaves intact the hypothesis that AT1 antagonists should not share all of the side effects of ACE inhibitors. It calls into question, however, whether or not they will share all their beneficial effects, and shows how little is known about how both act.

Table 6.1 Patient Characteristics

Patient Characteristic	
Sex, n	11 M/1 F
Age, y (mean±SD)	69±8
Primary diagnosis IHD/IDC, n	9/3
NYHA functional class II/III, n	6/6
LVEF, % (mean±SD)	23±9
Current smoker, n	3
On Diuretic, n	12
ACE inhibitor, n	12
Enalapril 10mg bd, n	10
Aspirin, n	8
Beta blocker, n	4
Statin, n	4
Nitrate, n	3
Digoxin, n	3
Calcium antagonist, n	2
Amiodarone, n	1
Oral hypoglycaemic	1
Body mass index, kg/m ² (mean±SD)	26±5
Cholesterol, mmol/l (mean±SD)	5.4±0.8
Triglycerides, mmol/l (mean±SD)	2.4±1.1

IHD = ischaemic heart disease; IDC = idiopathic dilated cardiomyopathy; NYHA = New York Heart Association; LVEF = left ventricular ejection fraction

Figure 6.1 Study design

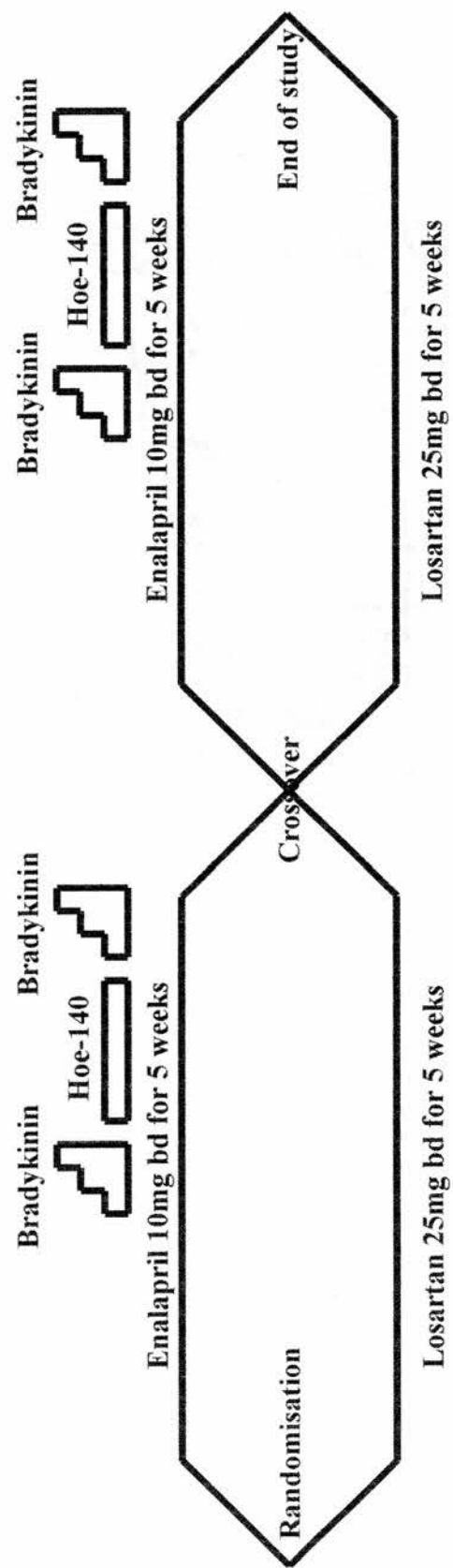


Figure 6.2 Heart rate during Hoe-140 infusion in 12 patients according to pre-treatment with enalapril or losartan (p=ns)

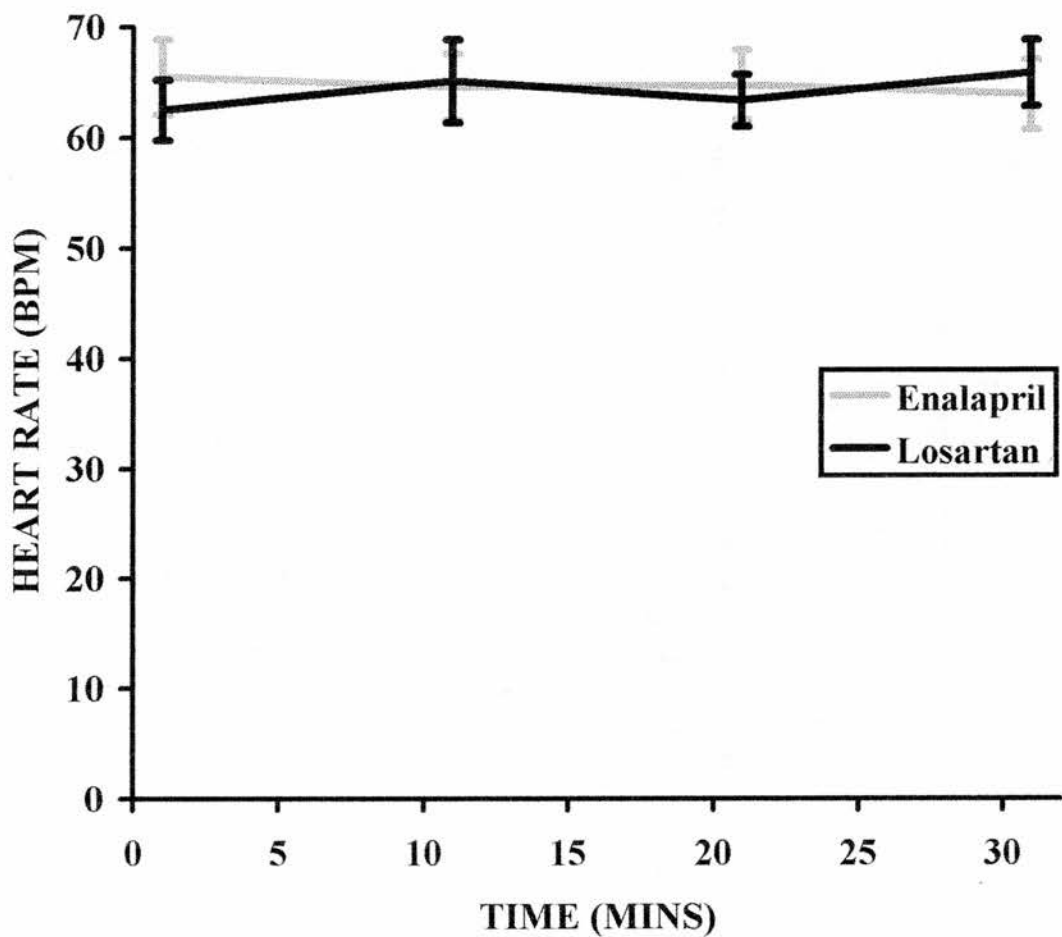


Figure 6.3 Mean blood pressure during Hoe-140 infusion in 12 patients according to pre-treatment with enalapril or losartan (p=ns)

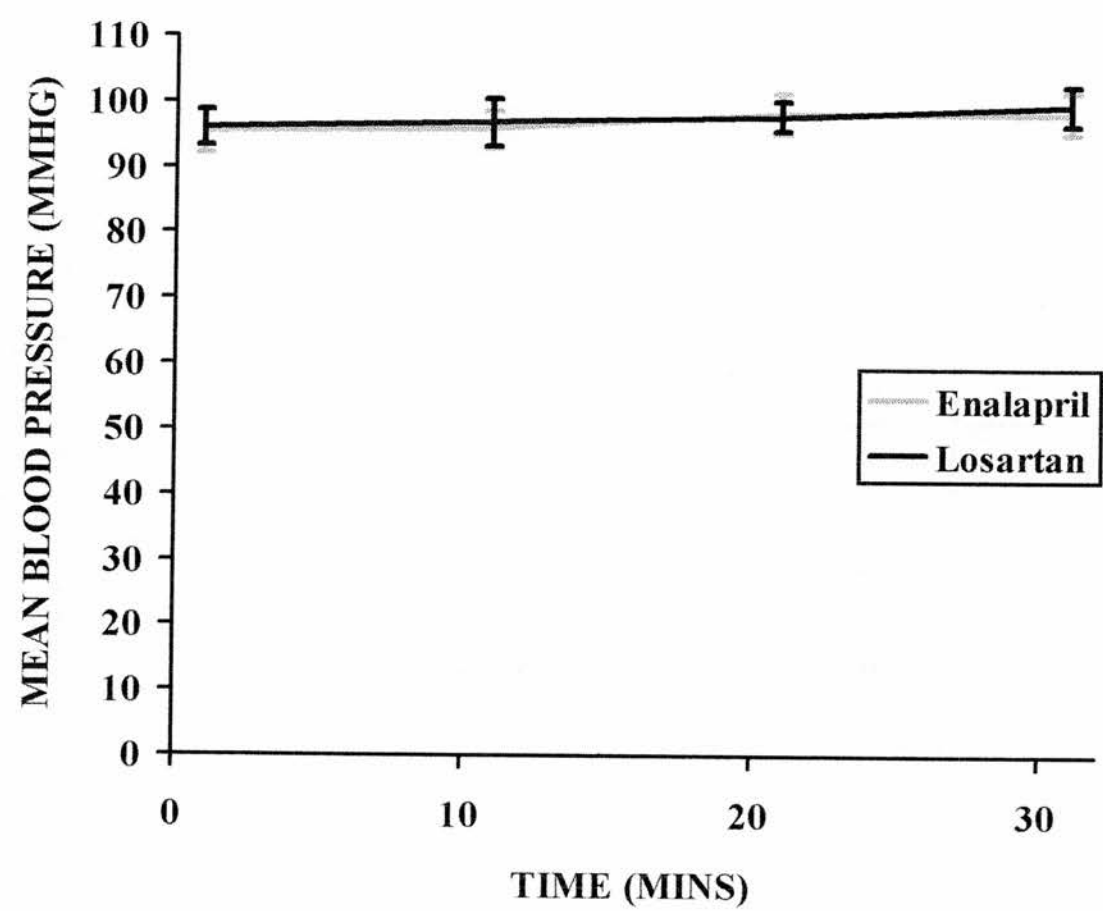


Figure 6.4 Effect of bradykinin on forearm blood flow before and after Hoe-140 (E = enalapril before Hoe-140; L = losartan before Hoe-140; E+H = enalapril after Hoe-140; L+H = losartan after Hoe-140)

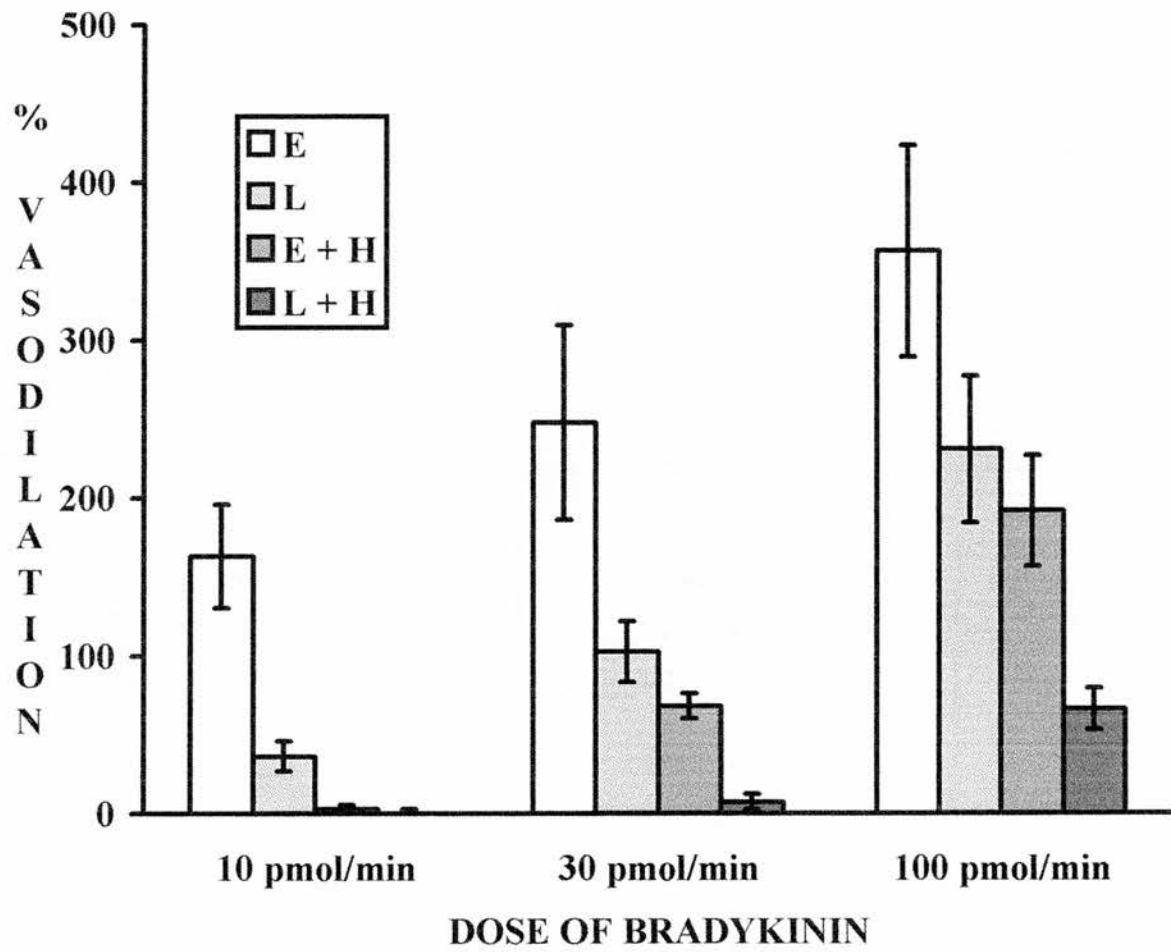


Figure 6.5 Effect of Hoe-140 on forearm blood flow

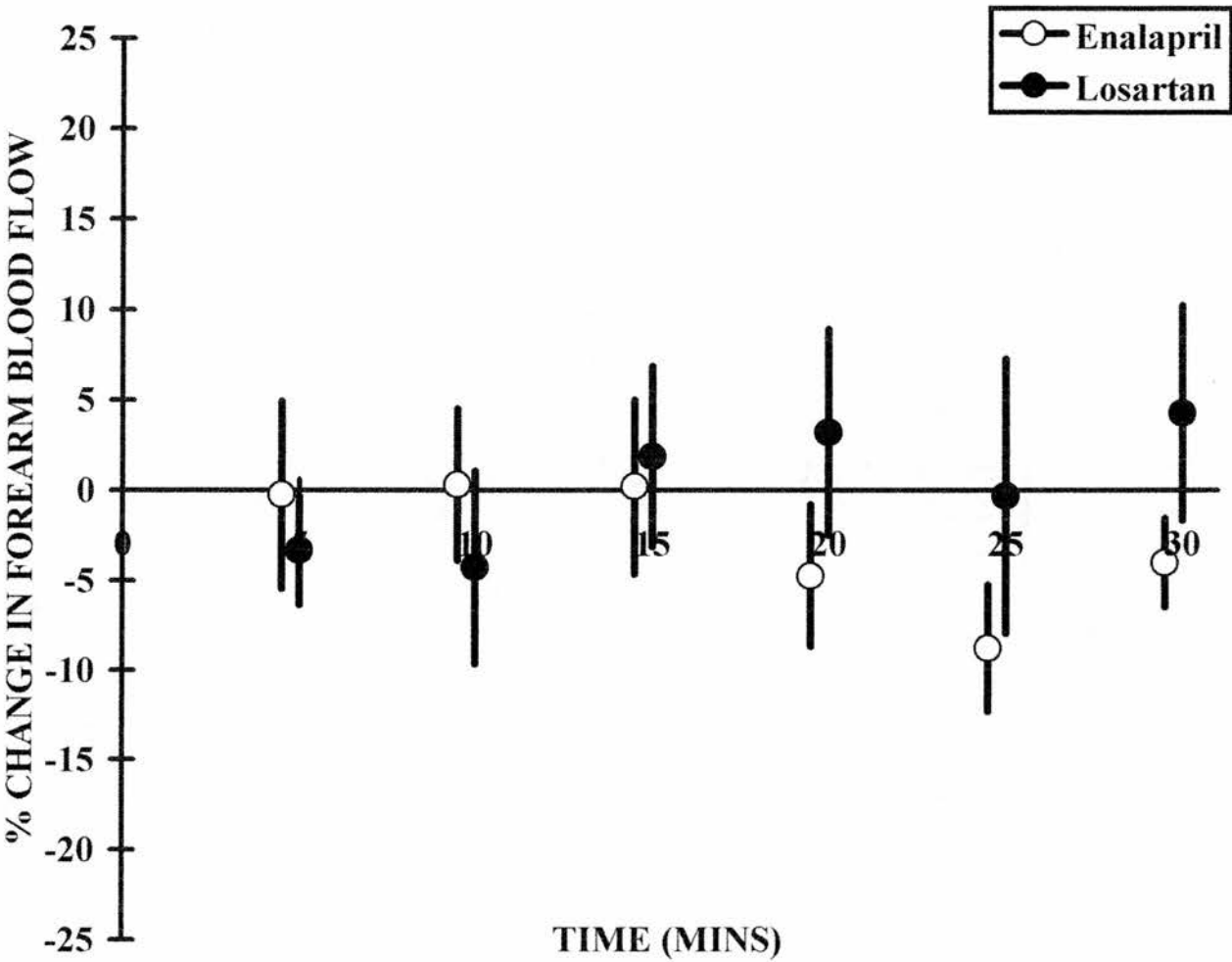
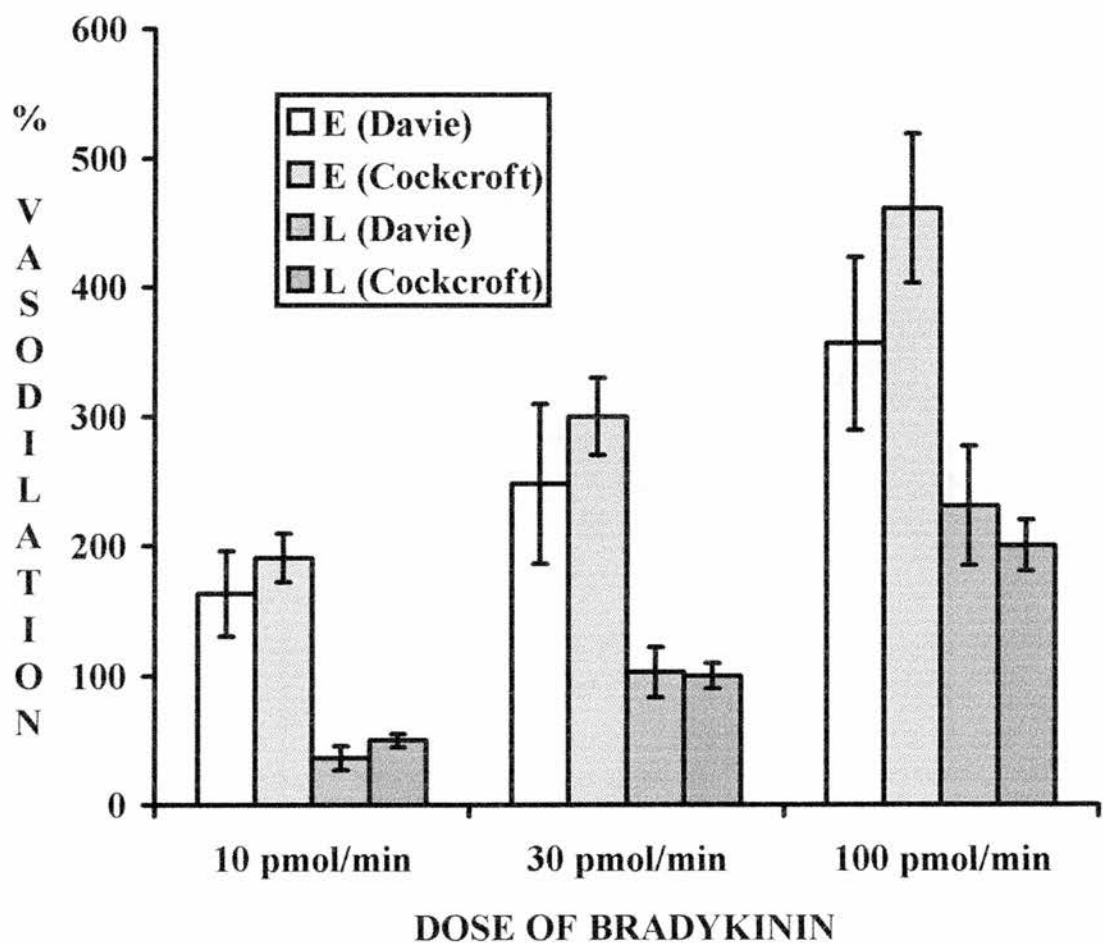


Figure 6.6 Comparison between our results with bradykinin in patients with heart failure and those of Cockcroft et al in healthy volunteers (E (Davie) = after 5 weeks of enalapril 10mg bd; E (Cockcroft) = after single dose of enalapril 10mg; L (Davie) = after 5 weeks of losartan 25mg bd; L (Cockcroft) = after single dose of losartan 20mg)



7.1 Introduction

Angiotensin-(1-7) was originally regarded as a biologically inactive degradation product of the renin-angiotensin-system (Nussberger *et al*, 1983; Tonnaer *et al*, 1983). An early study reported pressor effects in humans *in vivo*, but at such large doses that it could only be regarded as confirmation of biological inactivity (0.028% of the activity of angiotensin II) (Kono *et al*, 1986). It has since emerged that angiotensin-(1-7) is biologically active in the central nervous system (Campagnole-Santos *et al*, 1989), and indeed in the circulation (Osei *et al*, 1993). It has also emerged that angiotensin-(1-7) levels are not reduced by ACE inhibition (Welches *et al*, 1991), but may be increased, which implies that it is derived direct from angiotensin I (Campbell *et al*, 1991), and that the effect of angiotensin-(1-7) is potentiated by ACE inhibition (Porsti *et al*, 1994), which implies that it is inactivated by ACE (Chappell *et al*, 1998). It is therefore hypothesised that angiotensin-(1-7) may contribute towards the "non-angiotensin-II-ergic" effects of ACE inhibitors (Iyer *et al*, 1998), and even that this may be one way in which inhibitors of prostaglandin synthesis could interfere (Benter *et al*, 1993) with the action of ACE inhibitors (Hall *et al*, 1992). The situation is further complicated by the fact that angiotensin-(1-7) has been shown to interact closely with the effects of bradykinin. It is clear that it potentiates the effect of bradykinin (Paula *et al*, 1995), and even that its effects may be mediated by bradykinin (Abbas *et al*, 1997). Given that the effects of bradykinin are also potentiated by ACE inhibitors (Benjamin *et al*, 1989), this reiterates the potential importance of angiotensin-(1-7) (and bradykinin) in the effects of ACE inhibitors. It is hardly surprising that it has even been suggested that angiotensin-(1-7) may be acting as an ACE inhibitor itself (Li *et al*, 1997; Deddish *et al*, 1998; Roks

et al, 1999).

With a single exception (Kono *et al*, 1986), these studies were conducted in animals not humans, or were conducted *in vitro* rather than *in vivo*. Furthermore, despite the potential importance of angiotensin-(1-7) in the effects of ACE inhibitors, there has been no examination of its effects in heart failure, a syndrome in which the renin-angiotensin-system is of enormous importance and ACE inhibitors are extraordinarily clinically useful. We therefore sought to discover the effects of angiotensin-(1-7) in patients with heart failure treated with an ACE inhibitor, and any interaction with the effects of bradykinin.

7.2 Methods

7.2.1 Patients

The study was conducted with the approval of the West Ethics Committee and all patients gave written informed consent. Eight patients with chronic heart failure secondary to left ventricular systolic dysfunction confirmed by echocardiography were studied (left ventricular ejection fraction <40%). All patients were clinically stable on constant medication for 3 months, with no peripheral oedema or pulmonary congestion, and no uncontrolled hypertension, hypercholesterolaemia or diabetes mellitus. All patients were treated with an ACE inhibitor (5 patients enalapril 10mg bd, 1 patient lisinopril 10mg od, 1 patient captopril 25mg tds, 1 patient perindopril 4mg bd). All medication apart from aspirin was constant throughout the study. Patient characteristics are summarised in Table 7.1.

7.2.2 Treatment

Patients were studied after 14 days abstinence from aspirin. They took their usual medications apart from aspirin 6 hours before attendance on the day of study. Patients abstained from alcohol, tobacco and caffeine for 24 hours before each study and attended fasted for 6 hours. Bradykinin was infused at 3, 10 and 30 pmol/min for 3 minutes at each dose. Angiotensin-(1-7) was then infused at 5, 50, 500, 5000 and 50000 pmol/min for 6 minutes at each dose. These doses were almost completely arbitrary, as there are no previous reports of forearm studies with angiotensin-(1-7) in the literature. The doses were chosen to give as wide a dose-range from a single vial as possible, and to include potentially systemically active doses as well as potentially locally active doses. Finally, bradykinin was re-infused as before whilst a co-infusion of angiotensin-(1-7) at 50000 pmol/min continued.

7.2.3 Measurements

Forearm blood flow was measured by the technique of venous occlusion plethysmography as described in chapter 2. Plethysmographic recordings were made at 10 minute intervals during saline infusion and at 3-5 minute intervals during drug infusion. Blood pressure and pulse rate were manually recorded in the non-infused arm at 5-10 minute intervals throughout each study.

7.2.4 Drugs

Bradykinin (purity 99.4% by HPLC) was obtained from Clinalfa AG (Laufelfingen, Switzerland) and dissolved in normal saline. Angiotensin-(1-7) (purity 99.9% by HPLC) was obtained from Clinalfa AG and dissolved in normal saline.

Both drugs were always used within 2 hours of final preparation and destroyed thereafter.

7.2.5 Data analysis

All results are expressed as mean values with standard errors. All results were compared using two-tailed paired *t* tests. Differences were considered significant at a value of $p < .05$.

7.3 Results

7.3.1 General effects

Local infusion of bradykinin and angiotensin-(1-7) caused no adverse or systemic effects, and patients did not report any discomfort. Pulse rate (Figure 7.1), blood pressure (Figure 7.2), and forearm blood flow in the non-infused forearm did not change significantly during the initial infusions of bradykinin and angiotensin-(1-7). Nor was there any significant haemodynamic change during the co-infusion of bradykinin and angiotensin-(1-7), by the end of which, $0.78\mu\text{mol}$ had been administered in total, a dose which might reasonably have been expected to be systemically active.

7.3.2 Effect of bradykinin

Bradykinin caused marked vasodilation which was rapid in onset (~1 minute) and almost as rapid in offset (~5 minutes). There was obvious flushing of the whole forearm at the highest dose of bradykinin in the majority of cases, although no patient complained of this spontaneously. There was a clear dose-response relationship, with

peak vasodilation at the highest dose of 30 pmol/min and no sign of the development of significant tachyphylaxis (Figure 7.3).

7.3.3 Effect of angiotensin-(1-7)

There was evidence of slight vasoconstriction to angiotensin-(1-7) at 500 pmol/min ($4.3 \pm 3.0\%$) and 5000 pmol/min ($6.7 \pm 3.8\%$) but not at lower or higher doses. Even this effect was so small, however, that it was not significantly different from baseline (Figure 7.4)

7.3.4 Effect of angiotensin-(1-7) on bradykinin

Co-infusion of bradykinin and angiotensin-(1-7) after 30 minutes of angiotensin-(1-7) infusion gave very similar results to the initial infusion of bradykinin alone. If anything, there was a slight reduction in response, although this difference was not significant (Figure 7.1). This small but non-significant reduction could also have been due to slight tachyphylaxis.

7.4 Discussion

In this study in the forearm of patients with heart failure treated with an ACE inhibitor, we have shown that exogenous bradykinin causes vasodilation, that angiotensin-(1-7) has no significant effect on its own (except, if anything, a tendency to slight vasoconstriction) and that it does not potentiate the response to exogenous bradykinin (if anything, it inhibits it). These findings do not accord with previously reported findings in animals (many of them *in vitro* rather than *in vivo*) and therefore deserve further consideration.

7.4.1 Effect of bradykinin in heart failure

Our findings with bradykinin in this study replicate and validate our findings with bradykinin in chapters 5 and 6. The findings from this study confirm that bradykinin is a potent vasodilator in patients with heart failure, as it is in subjects without heart failure. As we have already observed it has recently been reported that responses to bradykinin are unimpaired in an animal model of heart failure (Su *et al*, 1998), but at the time of writing ours are the only studies of the effect of bradykinin in humans with heart failure in the entire literature (Davie *et al*, 1999).

7.4.2 Effect of angiotensin-(1-7) in heart failure

It was surprising that angiotensin-(1-7) had so little haemodynamic effect on its own and even more surprising that what effect it did have tended towards vasopressor effects rather than vasodepressor ones. At first sight this appears to contradict the substantial body of evidence reviewed in our introduction. Again, however, we point to the potential importance of the species gap. We find that our findings are consistent with the only three *in vivo* human studies in the literature. Firstly it was shown that a systemic infusion of angiotensin-(1-7) has pressor effects, albeit at a dose 24 times higher than the total dose we gave, and 3600 times higher than an equipotent dose of angiotensin II (Kono *et al*, 1986). Secondly, we are aware of two human studies that are cited as evidence that ACE inhibitors increase angiotensin-(1-7) levels. Lawrence *et al* (1990) appeared to show convincing elevations in angiotensin-(1-7) levels (and many other angiotensins) in hypertensive patients treated with ACE inhibitors. Enthusiasm for these results must be tempered by the fact that the comparison was with a case-control group of normotensive males,

rather than a comparison between the effects of treatment and non-treatment. Luque *et al* (1996) examined the effects of treatment, and appeared to show that angiotensin-(1-7) levels were increased by a last dose of captopril at the end of 6 months of treatment with captopril. Even that increased level, however, was not as high as the pre-treatment level, which was itself unaffected by the first dose of captopril. This was in marked contrast to the effects of captopril on angiotensin I levels. We contend that the available evidence suggests that angiotensin-(1-7) is biologically inactive in the circulation of man (certainly in the forearm of patients with heart failure treated with an ACE inhibitor). Our findings leave open the possibility (as alluded to in our introduction) that angiotensin-(1-7) is acting as an ACE inhibitor (given that our patients were already all on treatment with an ACE inhibitor). To test the hypothesis that angiotensin-(1-7) acts as an ACE inhibitor in humans it will obviously be necessary to test the effect of angiotensin-(1-7) in patients not treated with an ACE inhibitor.

7.4.3 Angiotensin-(1-7) and bradykinin in heart failure

We could find no evidence of any influence of quite massive doses of angiotensin-(1-7) on the response to bradykinin. It was important to demonstrate this in man, given the very clear demonstration that angiotensin-(1-7) potentiates the effects of bradykinin in animal models (Paula *et al*, 1995). Indeed, it has been reported that the effects of angiotensin-(1-7) itself are mediated by bradykinin. It has also been reported that angiotensin-(1-7) acts as an ACE inhibitor. It is obviously possible that an ACE inhibiting and/or bradykinin-potentiating effect was obscured by the fact that all our patients were already on an ACE inhibitor (if angiotensin-(1-7) is

an ACE inhibitor, it is certainly not as powerful a one as enalapril and related drugs) (Chappell *et al*, 1998). A lack of effect on the response to bradykinin is however compatible with the lack of effect of angiotensin-(1-7) alone which we have also demonstrated. Again, it does leave open the possibility that angiotensin-(1-7) might potentiate bradykinin by acting as an ACE inhibitor (if studied in patients not treated with an ACE inhibitor).

7.4.4 Summary

We have demonstrated that angiotensin-(1-7) has no significant effect on its own, or on the response to bradykinin, in the forearm arteries of patients with heart failure treated with an ACE inhibitor (who otherwise have quite impressive responses to bradykinin). These are patients in whom the renin-angiotensin-system is of considerable pathophysiological and therapeutic importance. Angiotensin-(1-7) has gained some currency as a potential mediator of "non-angiotensinergic" (or more properly, "non-angiotensin-II-ergic") effects of ACE inhibitors, and perhaps, other modulators of the renin-angiotensin-system, such as AT1 antagonists. Our results militate against any such role, and suggest both that it will be necessary to look elsewhere for mediators to explain the complexity of this system, and indeed that it may be a fruitless task to look for a pathophysiological role of angiotensin-(1-7) in humans with heart failure.

Table 7.1 Patient Characteristics

<i>Patient Characteristic</i>	
Sex, n	5 M/3 F
Age, y (mean +/- SD)	74 +/- 4
Weight, kg (mean +/- SD)	72 +/- 9
Primary diagnosis IHD/IDC, n	7/1
NYHA class II/III/IV, n	3/3/2
LVEF, % (mean +/- SD)	17 +/- 9
Current smoker, n	1
On Aspirin, n	6
ACE inhibitor, n	8
Diuretic, n	7
Digoxin, n	1
Nitrate, n	2
Calcium antagonist, n	3
Statin, n	1
Amiodarone	2
Baseline BP, mmHg (mean)	124/67

IHD = ischaemic heart disease; IDC = idiopathic dilated cardiomyopathy; NYHA = New York Heart Association; LVEF = left ventricular ejection fraction

Figure 7.1 Heart rate before, during and after angiotensin-(1-7) infusion

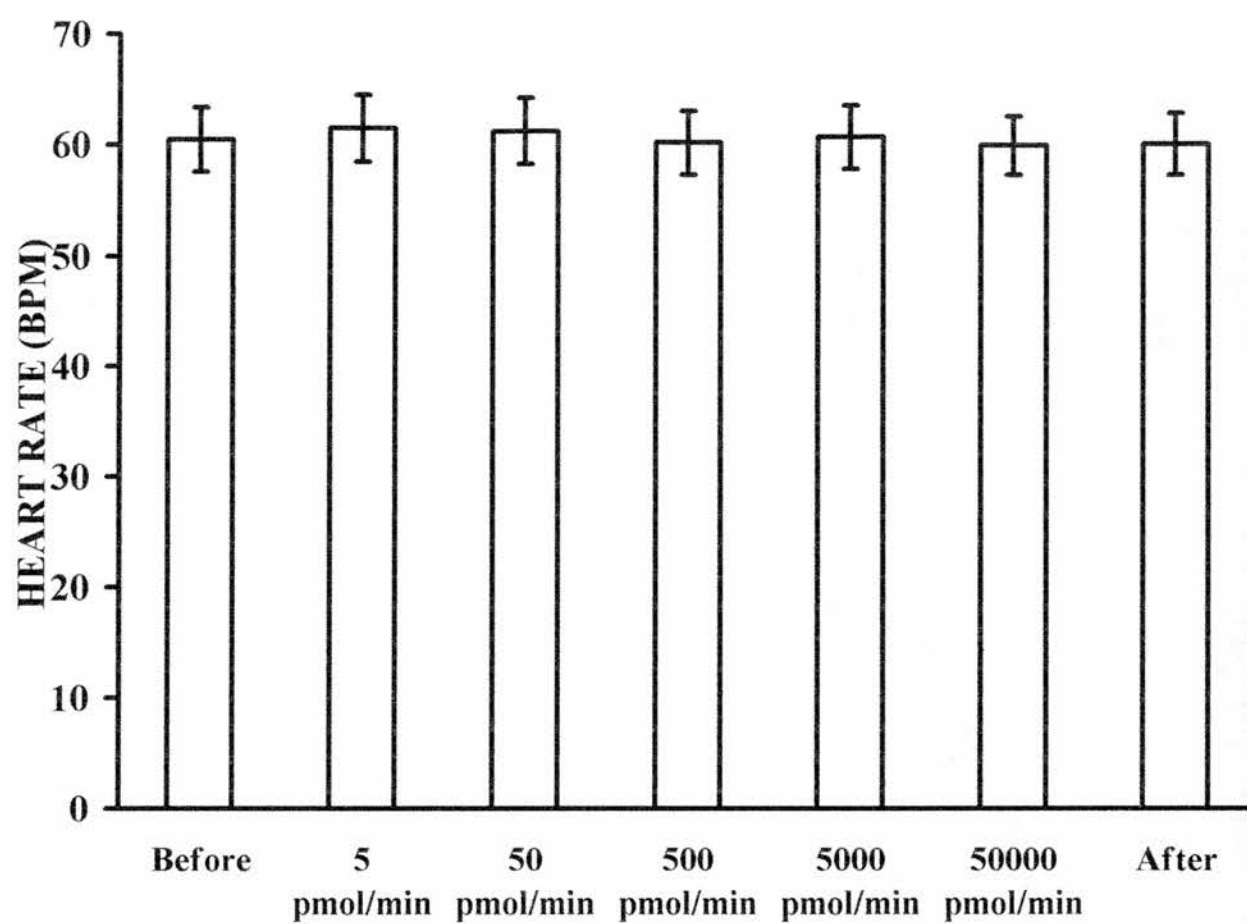


Figure 7.2 Mean blood pressure before, during and after angiotensin-(1-7) infusion

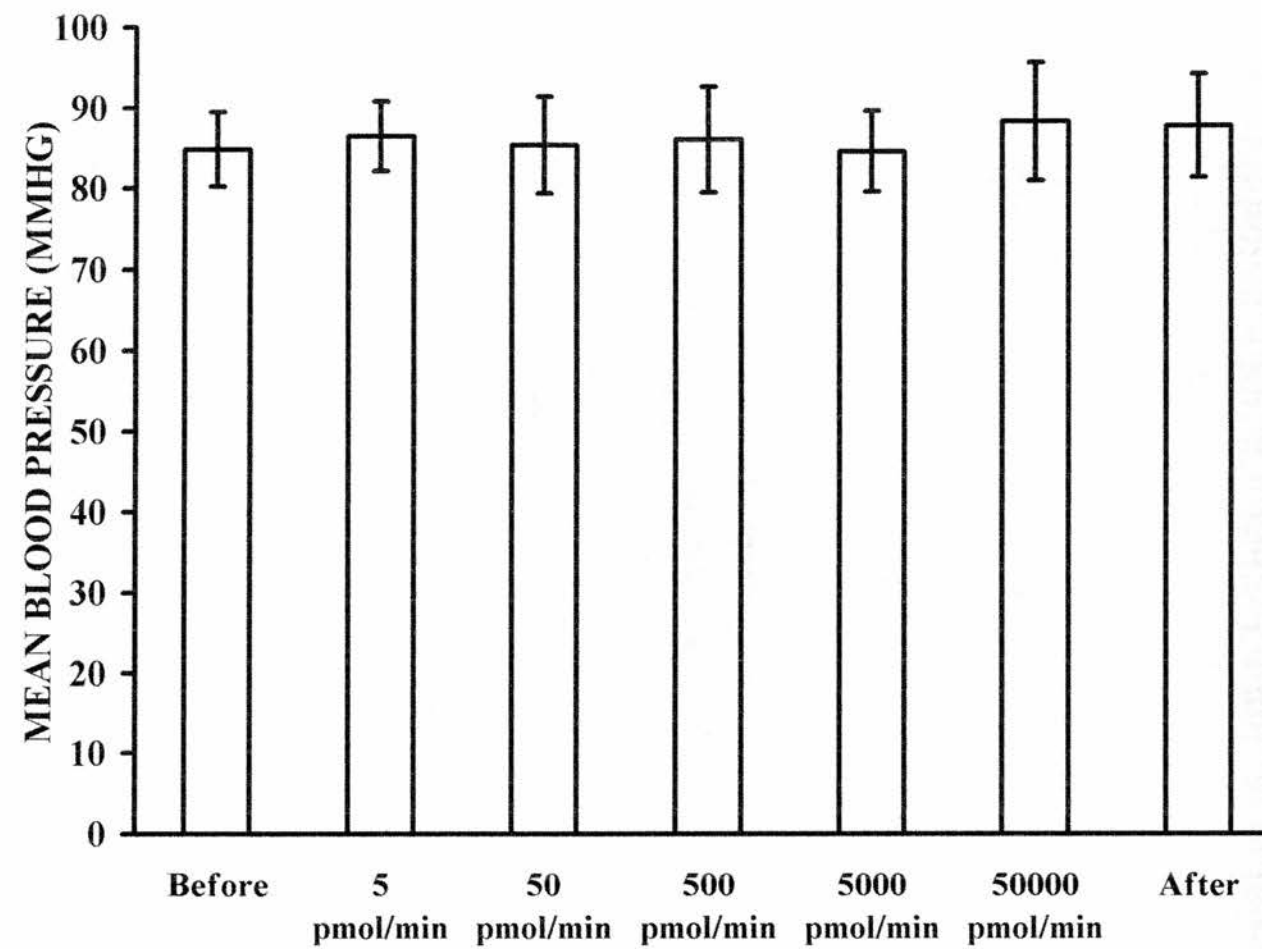


Figure 7.3 Effect of bradykinin on forearm blood flow prior to and during angiotensin-(1-7) infusion (B = prior to; B+A = during; p=ns, B versus B+A)

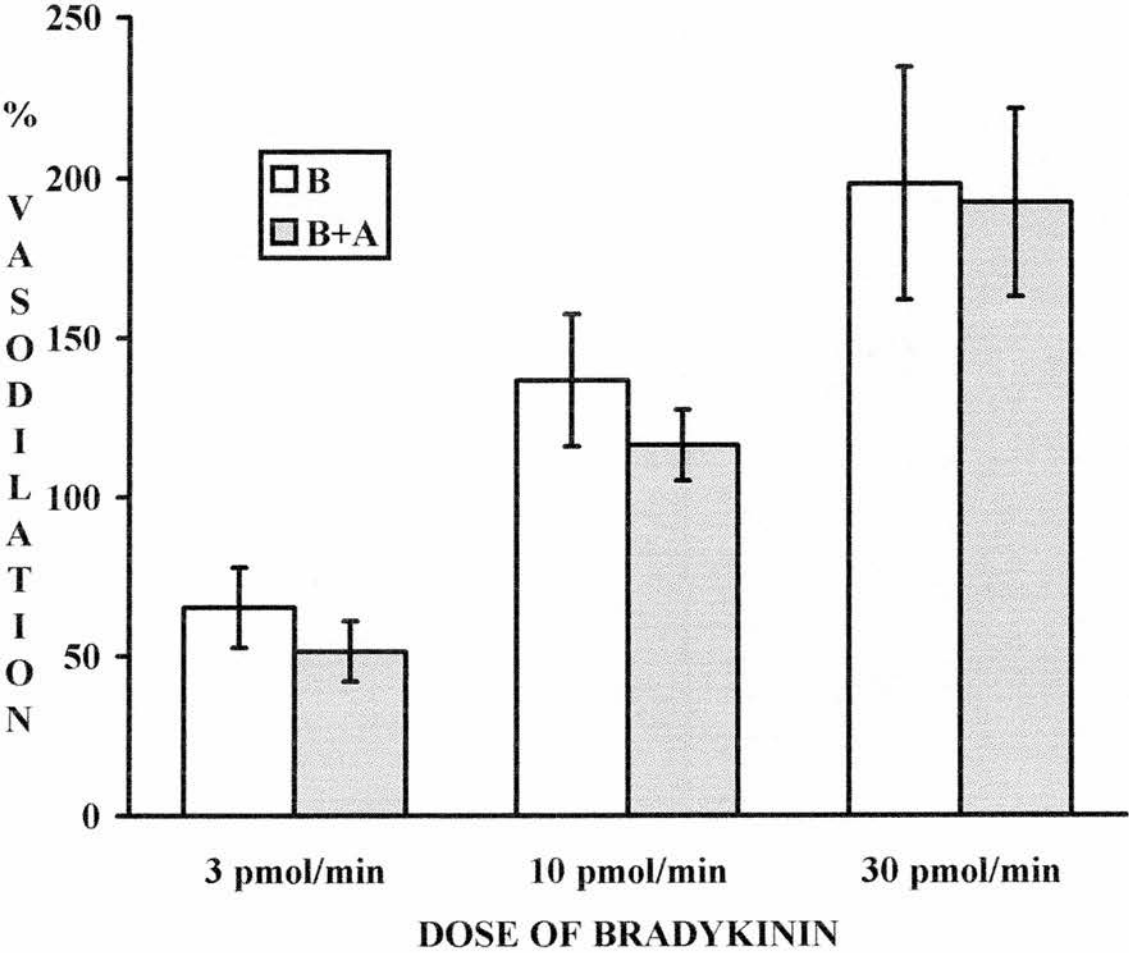
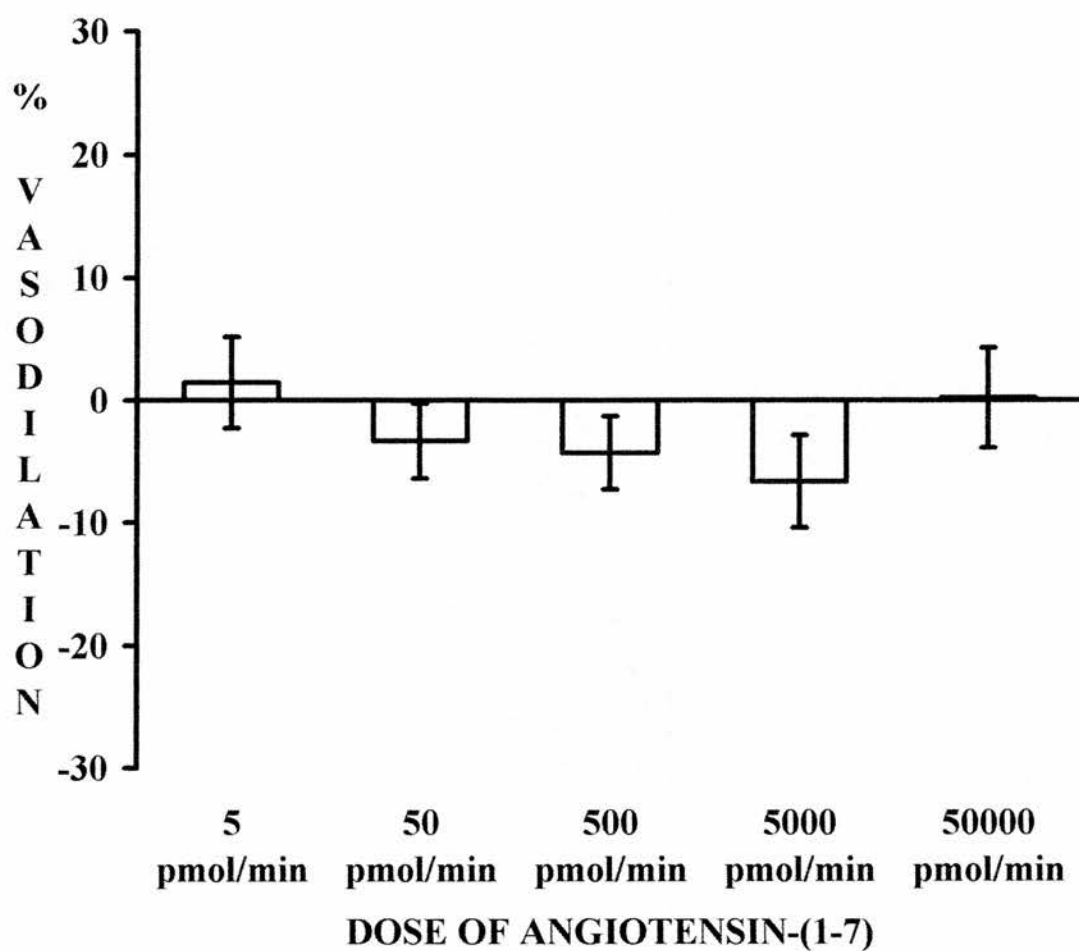


Figure 7.4 **Effect of angiotensin-(1-7) infusion (p=ns versus baseline)**



8.1 Introduction

It is not just vasoactive substances which are released by the endothelium. In particular, a number of components of the fibrinolytic system are also produced by endothelial cells, specifically, tissue-type plasminogen activator (TPA) and plasminogen activator inhibitor type I (PAI-1). TPA converts plasminogen to plasmin which is the active principle of fibrinolysis, and PAI-1 inhibits TPA itself (Figure 8.1). Endothelial cells also release von Willebrand Factor (VWF) which interacts more indirectly with the fibrinolytic system and the clotting cascade by acting as a carrier for factor VIII and by participating in the process of platelet adhesion to damaged endothelium. Recognition of the fact that elevated levels of VWF were associated with endothelial cell damage predated the concept of endothelial dysfunction by some years (Boneu *et al*, 1975). Indeed, TPA, PAI-1 and VWF have all been proposed as markers of endothelial dysfunction. In the case of TPA and PAI-1, however, the situation is complicated by their responsiveness to hormonal stimulation of the endothelium. In the case of TPA, release appears to be stimulated by bradykinin (Brown *et al*, 1997), and in the case of PAI-1, release appears to be stimulated by angiotensin II (Ridker *et al*, 1993). It is hardly surprising that a number of studies have attempted to examine the effect of ACE inhibitors and other modulators of the renin-angiotensin-system on TPA, PAI-1, VWF and D-dimers, the end product of the fibrinolytic system (Figure 8.1).

As shown in Table 8.1, these studies have been extremely heterogeneous in aims, design and methods, but despite this there are only four studies which have been completely "negative", and the other nine have been more or less "positive". None

showed any increase in any of these fibrinolytic parameters, so probably can be taken as evidence that ACE inhibitors tend to decrease some or all. This is assuming no publication bias, which seems unlikely, in the presence of so many "negative" results. This body of evidence has been cited as a possible explanation of the unexpected reduction in coronary events demonstrated with captopril in the SAVE study (Pfeffer *et al*, 1992). Only two of these studies have taken advantage of the opportunity to compare an ACE inhibitor with an AT1 antagonist (Goodfield *et al*, 1999; Brown *et al*, 1999). The first of these studies (in patients with heart failure) showed that losartan reduced PAI-1 antigen more than enalapril, and cited this as a possible explanation of the unexpected reduction in mortality with losartan demonstrated in the ELITE study (Pitt *et al*, 1997), but this was a study of the effects of a single dose only. The second study (in normals only) showed that quinapril but not losartan reduced PAI-1 activity and antigen, and that losartan but not quinapril reduced TPA antigen. We wanted to compare the effects of ACE inhibition and AT1 antagonism on plasma fibrinolytic variables in patients with heart failure over a rather longer period of time than the single dose previously studied. We therefore randomised patients with heart failure to double-blind cross-over treatment with enalapril and losartan (five weeks each) and measured the effect on fibrinolytic parameters.

8.2 Methods

8.2.1 Patients

The study was conducted with the approval of the West Ethics Committee and all patients gave written informed consent. Twelve patients with chronic heart failure secondary to left ventricular systolic dysfunction confirmed by echocardiography were

studied (left ventricular ejection fraction <40%). All patients were treated with an ACE inhibitor at baseline (enalapril 10mg bd in the majority). All medication apart from ACE inhibitor/AT1 antagonist aspirin was constant throughout the study. Patient characteristics are shown in Table 6.1

8.2.2 Randomisation

Patients were randomised to double-blind cross-over treatment with enalapril 10mg bd for 5 weeks followed immediately by losartan 25mg bd for 5 weeks, or *vice versa*. At the end of each 5 weeks patients attended for study, having abstained from all aspirin therapy for 14 days. Patients took all their other usual medication including study treatment at 8am before attendance on the day of study at 2pm. It has long been known that plasma fibrinolytic parameters exhibit significant diurnal variation (Angleton *et al*, 1989). Although we were not in a position to examine this variability, it was clearly important that all our studies were performed at the same time.

8.2.3 Measurements

Supine blood pressure and pulse rate were manually recorded using a mercury sphygmomanometer. After lying supine for 20 minutes, 10ml of blood was withdrawn from the antecubital fossa of the right forearm, collected into sodium citrate tubes and kept on ice before being centrifuged at 3000rpm for 20 minutes at 4°C. Platelet-free plasma was then decanted and stored at -80°C before assay. PAI-1 activity was measured with a commercially available chromogenic substrate assay (Coatest PAI; Chromogenix, Epsom) and compared with a pool of normal results to give the result

as a percentage of that normal pool. TPA was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) from Biopool AB, Umea, Sweden. Von Willebrand factor (VWF) antigen was measured with an in-house ELISA using rabbit anti-human polyclonal antibodies from DAKO plc, High Wycombe. Fibrin D-dimer was measured with a commercially available ELISA from Biopool AB, Umea, Sweden. These are all routine assays, well-established in our department (Sattar *et al*, 1999).

8.2.4 Data analysis

All results are expressed as mean values with standard errors. All results were compared using two-tailed paired *t* tests. Differences were considered significant at a value of $p < .05$.

8.3 Results

8.3.1 General effects

Haemodynamic and fibrinolytic parameters are shown in Table 8.2. There was no significant difference in pulse rate and blood pressure between either of the studies (although there was a trend towards a lower blood pressure in the enalapril study compared with the losartan study).

8.3.2 Fibrinolytic parameters

Fibrinolytic parameters are shown in Table 8.2 and Figure 8.2. There was no significant difference in any of the fibrinolytic parameters studied between either of the studies, and all were within the normal range for our laboratory. Despite the

changes previously demonstrated, we found no difference in TPA or PAI-1. Despite its association with endothelial function, we found no difference in VWF. Despite its status as an endpoint of the fibrinolytic system, we found no difference in D-dimer.

8.4 Discussion

We have shown that there is no significant difference in plasma fibrinolytic variables after 5 weeks of enalapril 10mg bd and after 5 weeks of losartan 25mg bd, in patients with heart failure. In particular, we have failed to show any difference whatsoever in PAI-1 activity. This is despite the fact that it has previously been shown that a single dose of losartan 50mg reduces PAI-1 antigen more than a single dose of enalapril 10mg in patients with heart failure (Goodfield *et al*, 1999). It is also despite the fact that it has recently been shown that it is angiotensin-(3-8) (angiotensin IV) acting at the angiotensin type 4 (AT4) receptor which mediates PAI-1 release by endothelial cells (Kerins *et al*, 1995; Gesualdo *et al*, 1999). More recently it has been shown that 10 days of quinapril 40mg bd reduced PAI-1, but 10 days of losartan 50mg bd reduced TPA antigen (Brown *et al*, 1999). These findings appear to be somewhat contradictory, and the apparent discrepancy deserves closer consideration.

8.4.1 Acute versus chronic effects

If acute AT1 antagonism reduces PAI-1 more than acute ACE inhibition, it would be expected that chronic AT1 antagonism would also reduce PAI-1 more than chronic ACE inhibition. In fact there was no difference. It is impossible to say whether this was because the ACE inhibitor and the AT1 antagonist were equally effective or because they were equally ineffective. Assuming that their acute effects

were as previously demonstrated, it is impossible to say whether with chronic ACE inhibition PAI-1 falls further or with chronic AT1 antagonism PAI-1 drifts up again. It certainly suggests that there is a potential distinction to be made between the acute and chronic effects of AT1 antagonism and ACE inhibition. It is already well known that chronic effects of ACE inhibitors may differ from their acute effects (Sharpe *et al*, 1980). It is not known to what extent the chronic effects of AT1 antagonists might differ from their acute effects. This only emphasises how important it is that acute studies are complemented by more chronic studies. Under these circumstances, to find that there is a discrepancy between the acute and chronic effects of AT1 antagonism and ACE inhibition on PAI-1 in patients with heart failure is hardly surprising.

8.4.2 Diurnal variation

It is equally possible that the difference between our findings and previous studies is accounted for by some difference in timing relative to the diurnal variation of plasma fibrinolytic variables. We took care, however, to make sure that our timing was consistent from study to study, and not more than an hour or two different from previous studies. Diurnal variation of plasma fibrinolytic variables is not so rapid as make much difference because of a few hours either way during the day.

8.4.3 Drug effects

It is obviously possible that the discrepancy between this study and the previous one is accounted for by some factor other than timing (although timing is the most obvious difference). It is interesting to note that, whereas we discontinued all

aspirin therapy 14 days before study, there is no mention of this in the previous study (8 out of 12 of our patients were treated with aspirin, compared to 13 out of 20 in the previous study). This actually raises the tantalising possibility that the difference between our results and previous results may be due to an interaction with the effects of aspirin. We used exactly the same drugs (enalapril and losartan) and exactly the same sort of patients (patients with stable chronic heart failure), but there is a difference in dosing. Whereas we used enalapril 20mg a day and losartan 50mg a day, the previous study used a single dose of enalapril 10mg and losartan 50mg. The doses we used were based on the evidence of the best available clinical trials. The dose of enalapril came from the Studies of Left Ventricular Dysfunction (The SOLVD Investigators, 1991; 1992). The dose of losartan came from ELITE (Pitt *et al*, 1997). This raises the very real possibility that the difference between losartan and enalapril shown in the previous trial is dose-dependent, the difference being between the response to a whole day's dose of losartan and half a day's dose of enalapril. Of course, it is difficult to compare a long-acting drug with a shorter-acting drug, either acutely or chronically. Even so, it is hardly surprising that a study effectively comparing the same dose of losartan with double the dose of enalapril has redressed the balance. In this context, it is very interesting that a study comparing the effects of a very large dose of quinapril for 10 days with double the dose of losartan for 10 days did find a difference between them. Despite this, the authors were unable to attach any qualitative clinical significance to the fact that quinapril reduced PAI-1 more and losartan reduced TPA more (Brown *et al*, 1999). Whatever the case, it is clearly important that the message that AT1 antagonism has more beneficial effects on plasma fibrinolytic variables than ACE inhibition does not go uncontradicted.

Figure 8.1 Interaction of the endothelium with the plasma fibrinolytic system

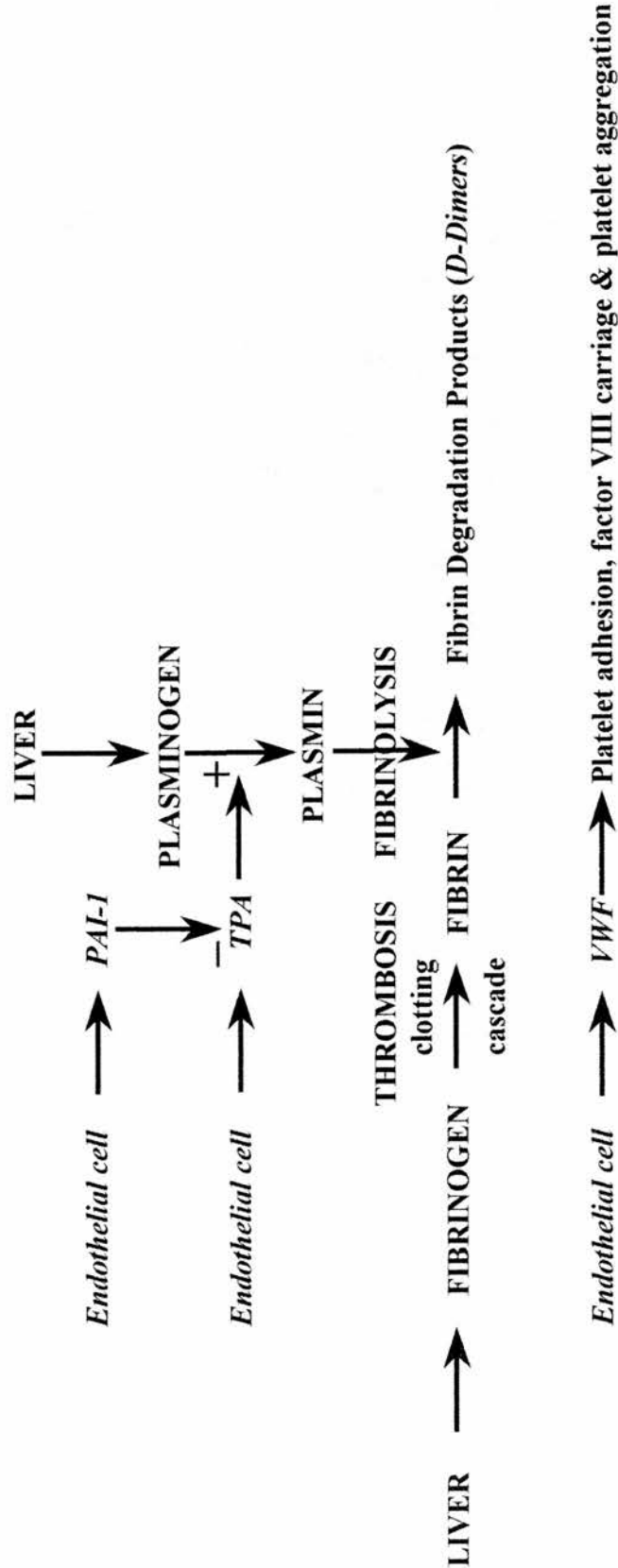


Table 8.1 Studies with ACE inhibitors and plasma fibrinolytic parameters

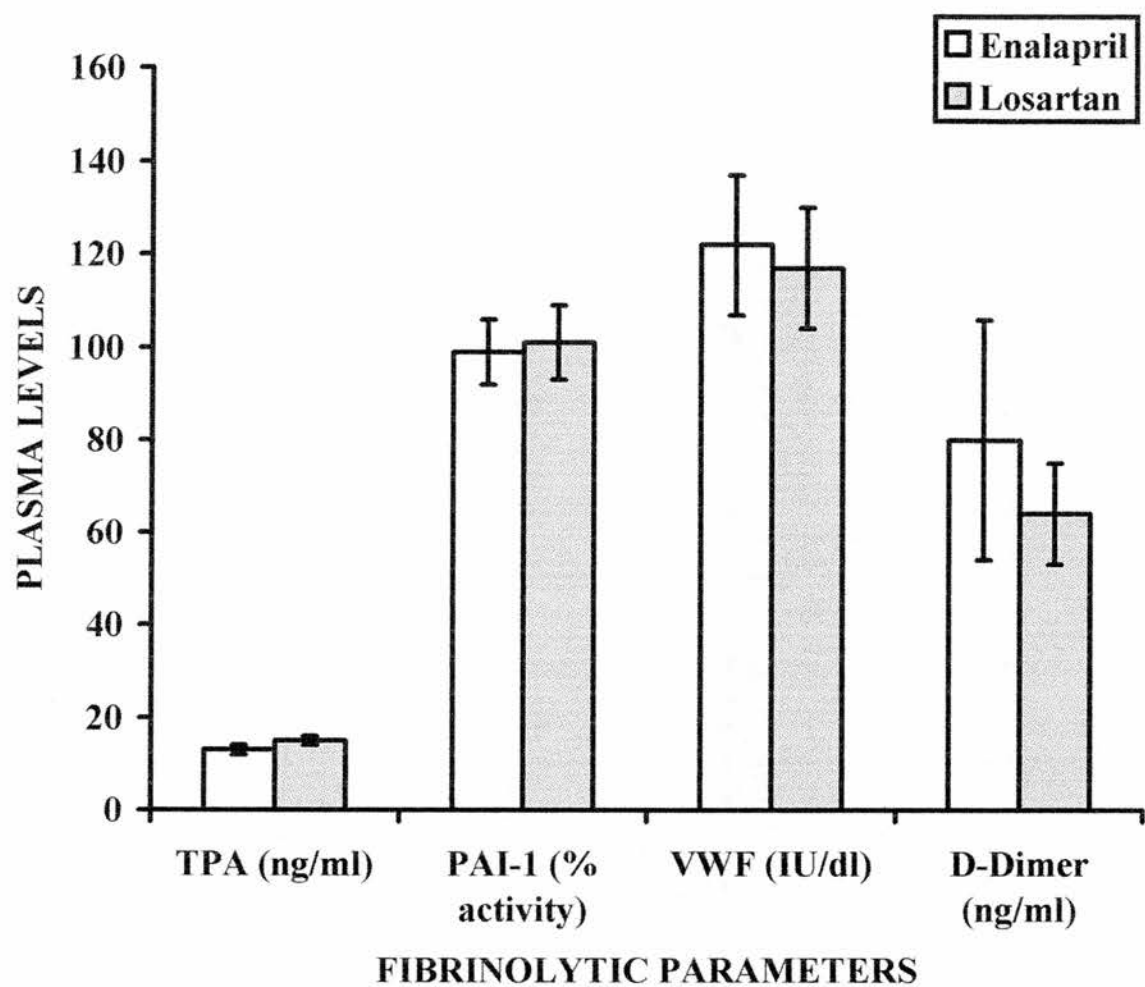
<i>1st author</i>	<i>Year</i>	<i>ACEI</i>	<i>Patients</i>	<i>Length</i>	<i>Randomised</i>	<i>n treated</i>	<i>PAI-1 activity</i>	<i>PAI-1 antigen</i>	<i>TPA activity</i>	<i>TPA antigen</i>	<i>VWF</i>
Jansson	1993	Enalapril	post-MI	3 months	yes	41	=	=	-	↓	-
Wright	1994	Captopril	post-MI	4 weeks	yes	15	↓	=	-	↓	-
Zehetgruber	1996	Lisinopril	CAD	12 weeks	no	12	=	=	-	-	-
Moriyama	1997	Captopril	post-MI	48 hours	no	14	↓	-	-	↓	-
Pedersen	1997	Trandolapril	post-MI	1 year	yes	28	-	=	-	=	-
Soejima	1997	Imadipril	post-MI	4 weeks	yes	15	↓	↓	-	=	-
Vaughan	1997	Ramipril	post-MI	14 days	yes	60	↓	↓	-	=	-
Brown	1998	Quinapril	Normals	1 month	no	9	↓	↓	-	=	-
Sayer	1998	Quinapril	CAD	24 hours	no	20	=	-	=	-	-
Brown	1999	Quinapril	Normals	1 month	no	25	↓	↓	-	=	-
Goodfield	1999	Enalapril	CHF	6 hours	yes	20	=	↓	=	↓	-
Lottermoser	1999	Captopril	Normals	2 weeks	no	10	=	=	=	=	=
Sakata	1999	Enalapril	HBP	3 months	no	22	↓	-	-	=	-

ACEI = ACE inhibitor; MI = myocardial infarction; CAD = coronary artery disease; CHF = congestive heart failure; HBP = hypertension
↓ = decreased; = = unchanged; - = not studied

Table 8.2 Effect of losartan and enalapril on systemic haemodynamics and fibrinolytic parameters

	Enalapril	Losartan
Heart rate, bpm	67±5	63±3
Mean arterial pressure, mmHg	92±4	99±4
TPA antigen, ng/ml	13±1	15±1
PAI-1 activity, %	99±7	101±8
VWF, IU/dl	122±15	117±13
D-dimer, ng/ml	80±26	64±11

Figure 8.2 Effect of losartan and enalapril on fibrinolytic parameters



9.1 Introduction

A consistent feature of vascular biology over the last few years has been the finding that stimulated nitric oxide release (the syndrome of endothelial dysfunction) is reduced in heart failure (Kaiser *et al*, 1989; Treasure *et al*, 1990; Kubo *et al*, 1991; Drexler *et al*, 1992). Despite this, attempts to define the level of basal nitric oxide release in heart failure have been somewhat inconsistent, partly because they have relied on indirect methods. The earliest efforts used L-NG-monomethylarginine (L-NMMA), an inhibitor of nitric oxide synthesis. These gave results that implied that basal nitric oxide release was increased (Drexler *et al*, 1992, Habib *et al*, 1994), unchanged (Kubo *et al*, 1994) or reduced (Katz *et al*, 1996). Plasma nitrate levels also gave results which implied that basal nitric oxide release was increased (Winlaw *et al*, 1994). Previously, exhaled nitric oxide levels had implied that basal nitric oxide release was reduced (Adachi *et al*, 1997), but very recently it has been conclusively demonstrated that exhaled nitric oxide is not vascular endothelial in origin, but alveolar epithelial (Sartori *et al*, 1999). Over the same period of time, molecular biology has been attempting to disentangle the intricacies of nitric oxide synthase (NOS) gene expression in heart failure, the results of which have been reasonably consistent. The majority of studies have found reduced expression of the constitutional form of NOS (endothelial cell NOS, ecNOS or NOS III), both in endothelial cells (Smith *et al*, 1996; Wang *et al*, 1997; Gaballa *et al*, 1999) and in myocardial cells (Drexler *et al*, 1998). Similarly the majority of studies have found increased expression of inducible NOS (iNOS or NOS II), both in myocardial cells (Haywood *et al*, 1996; Satoh *et al*, 1997; Drexler *et al*, 1998) and in skeletal muscle (Riede *et al*, 1998). What this actually means for overall NOS activity and overall

nitric oxide release is obviously very difficult to say. Very recently, the issue appears to have been settled once and for all. Katz *et al* (1999) used a radioisotope labelled tracer to track actual whole body nitric oxide synthesis. This showed that basal *and* stimulated (by exercise) nitric oxide synthesis were both reduced in patients with heart failure.

At the same time as the above, the idea has emerged that angiotensin converting enzyme (ACE) inhibitors might actually help restore impaired endothelial function (i.e., stimulated and possibly basal nitric oxide release) in heart failure (Mancini *et al*, 1996). This could be an important difference between ACE inhibitors and angiotensin II type I receptor (AT1) antagonists, especially as it has been suggested that the improvement in endothelial function from an ACE inhibitor might be bradykinin-dependent. It has also been suggested that the improvement in endothelial function from an ACE inhibitor might actually be blocked by aspirin. Nakamura *et al* (1994) found that co-infusion of enalaprilat enhanced vasodilation to acetylcholine before, but not after, pre-treatment with aspirin. It was particularly for this reason that we were interested in some sort of comparison of endothelial function between patients with heart failure treated with an ACE inhibitor and an AT1 antagonist, whilst off aspirin. Any difference between the two might represent a potential substrate for an interaction with aspirin by one, but not the other. For the purposes of this study patients with heart failure were therefore randomised to double-blind cross-over treatment with an ACE inhibitor and an AT1 antagonist (five weeks each) and plasma nitrate/nitrite was measured.

9.2 Methods

9.2.1 Patients

The study was conducted with the approval of the West Ethics Committee and all patients gave written informed consent. Twelve patients with chronic heart failure secondary to left ventricular systolic dysfunction confirmed by echocardiography were studied (left ventricular ejection fraction <40%). All patients were treated with an ACE inhibitor at baseline (enalapril 10mg bd in the majority). All medication apart from ACE inhibitor/AT1 antagonist and aspirin was constant throughout the study. Patient characteristics are shown in Table 6.1

9.2.2 Randomisation

Patients were randomised to double-blind cross-over treatment with enalapril 10mg bd for 5 weeks followed immediately by losartan 25mg bd for 5 weeks, or *vice versa*. At the end of each 5 weeks patients attended for study, having abstained from all aspirin therapy for 14 days. Patients took all their other usual medication including study treatment (including nitrate treatment if applicable) at 8am before attendance on the day of study at 2pm having fasted for 6 hours (but otherwise having taken their normal diet).

9.2.3 Measurements

Supine blood pressure and pulse rate were manually recorded using a mercury sphygmomanometer. After lying supine for 20 minutes, 10ml of blood was withdrawn from the antecubital fossa of the right forearm, collected into heparinised tubes and kept on ice before being centrifuged at 3000rpm for 20 minutes at 4°C. Platelet-free

plasma was then decanted and stored at -80°C before assay. Total nitrite plus nitrate concentration was assayed as described by Phizackerley & Al-Dabbagh (1983). To reduce nitrate to nitrite, supernatant or standards were incubated at room temperature in the presence of *Klebsiella pneumoniae* under anaerobic conditions. Total nitrite in the supernatant was subjected to the Griess reaction (Green *et al*, 1982) and assayed spectrophotometrically. Data are reported as the sum of nitrite plus nitrate. Normal values in our laboratory were 5-15 mmol/L. The limit of detection was 0.1 mmol/L.

9.2.4 Data analysis

All results are expressed as mean values with standard errors. All results were compared using two-tailed paired *t* tests. Differences were considered significant at a value of $p < .05$.

9.3 Results

9.3.1 General effects

Haemodynamic parameters are shown in Table 8.2. There was no significant difference in pulse rate and blood pressure between either of the studies (although there was a trend towards a lower blood pressure in the enalapril study compared with the losartan study).

9.3.2 Effect on plasma nitrate/nitrite concentrations

Plasma nitrate/nitrite levels are shown in Figure 9.1. There was no significant difference in nitrate/nitrite levels between either of the studies (although there was a trend towards higher levels in the enalapril study compared with the losartan study).

There was no evidence whatsoever of any association between plasma nitrates/nitrite levels and blood pressure. There was also no significant difference in nitrate/nitrite levels between the 3 patients who were on habitual treatment with nitrates and the 9 patients who were not.

9.4 Discussion

We have shown that there is no significant difference in nitrate/nitrite levels after 5 weeks of enalapril 10mg bd and after 5 weeks of losartan 25mg bd, in patients with heart failure. To our knowledge this is the first time that any such comparison has been reported, especially in humans with heart failure. The data from animal models of endothelial dysfunction is somewhat contradictory.

9.4.1 Animal versus human studies

Some studies indicate that ACE inhibitors and AT1 antagonists improve endothelial function equally (Rodrigo *et al*, 1997; Hoshino *et al*, 1998; Teisman *et al*, 1998). Other studies indicate that ACE inhibitors improve endothelial function more than AT1 antagonists (Berkenboom *et al*, 1997). In some cases this appears to be because of a bradykinin dependent mechanism (Berkenboom *et al*, 1997). In humans there is a study which shows that quinapril and losartan both improved postprandial endothelial dysfunction (Wilmink *et al*, 1999), although quinapril did so more than losartan. There is also an ongoing study which has been reported to show that quinapril improves endothelial dysfunction in patients with coronary disease, but that enalapril and losartan do not (Anderson, 1999).

9.4.2 Blood pressure and aspirin therapy

The small but not significant difference in nitrate/nitrite levels we recorded between enalapril and losartan might be taken as confirmation of a prejudice that ACE inhibitors are in some way superior to AT1 antagonists, especially as it went the same way as a small difference in blood pressure. This was despite the fact that there was no evidence of any association between nitrate/nitrite levels and blood pressure. It would obviously be interesting to see if there was any association between nitrate/nitrite levels and aspirin therapy, especially as it has been suggested that ACE inhibitors might improve endothelial function by bradykinin-mediated, prostaglandin-dependent mechanism. It was for this reason that we withheld aspirin therapy, to make sure that it was not a confounding influence.

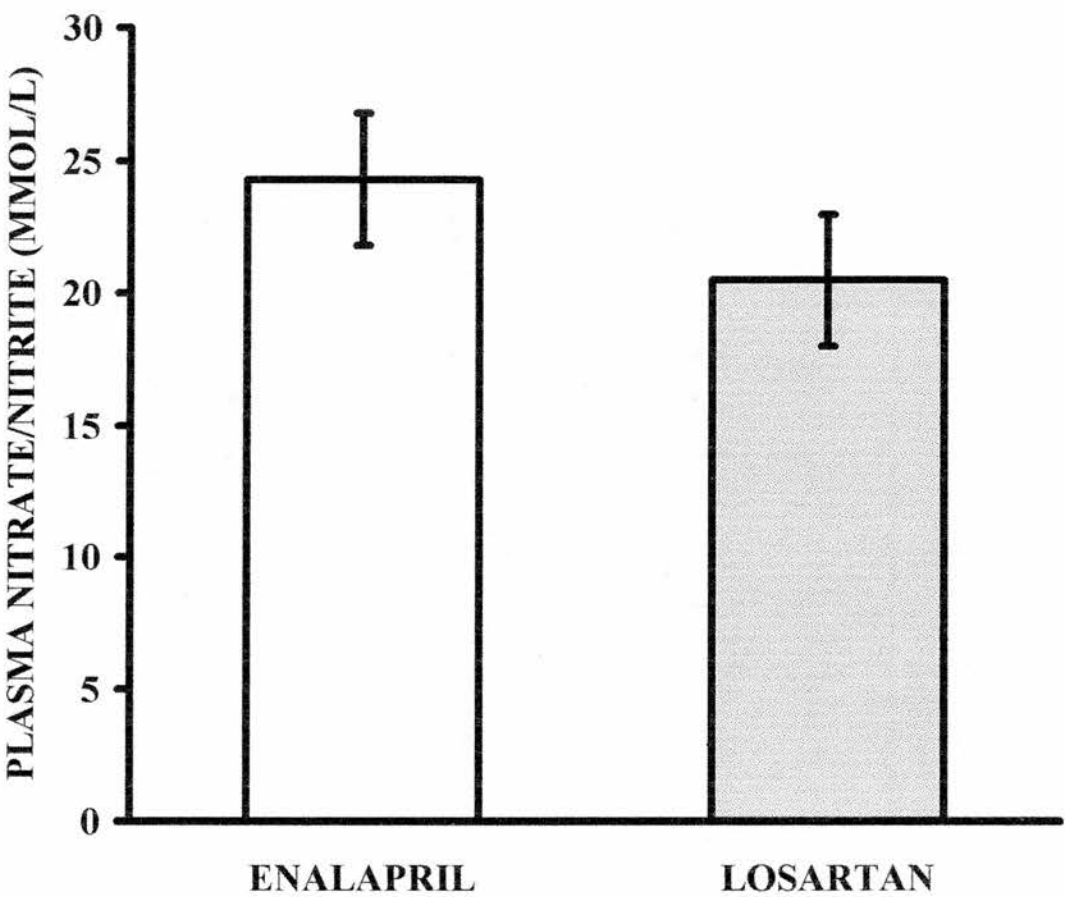
9.4.3 Superoxide pathway of endothelial dysfunction

Recently evidence has come about that one pathway of development of endothelial dysfunction might relate to angiotensin II activation of endothelial NADPH-oxidase to produce superoxide which neutralises nitric oxide (Rajagopalan *et al*, 1996). This is obviously a mechanism by which ACE inhibitors and AT1 antagonists might influence endothelial function directly, with an obvious effect on plasma nitrate/nitrite concentrations. The absence of any significant difference between ACE inhibitors and AT1 antagonists with respect to nitrate/nitrite concentrations does not tell us whether this pathway is important or not, but does imply that if it is, it is probably due to angiotensin II alone, acting at the AT1 receptor alone.

9.4.4 Summary

The absence of any significant difference in nitrate/nitrite levels between patients with heart failure treated with enalapril and the same patients treated with losartan can hardly be taken to prove that there is no difference in the effects of these agents on endothelial function. Although it is reasonable to postulate that there might be a correlation between nitrate/nitrite levels and more conventional measures of endothelial function in the form of endothelium-dependent vasodilatation, we are not aware of any evidence that there is such a correlation. It would have been better to look at endothelium-dependent vasodilatation in our patients as well, but we did not have the opportunity to do so, and we did have the opportunity to look at nitrate/nitrite levels. We found no evidence that there is any difference between enalapril and losartan in this regard, at least as far as patients with heart failure are concerned. The finding should perhaps be regarded as hypothesis-generating only, and subjected to further experimental scrutiny with more conventional measures of endothelial function, especially given the lack of human data and the inconsistency of the animal data alluded to above.

Figure 9.1 Plasma nitrate/nitrite levels in patients with heart failure treated with enalapril & losartan



10.1 Chapter 3 Effect of arachidonic acid in heart failure

The studies described in chapter 3 demonstrate that arachidonic acid is vasodilating in forearm resistance arteries in healthy volunteers and in patients with heart failure treated with an ACE inhibitor. This is a very similar result to the effect of arachidonic acid in the hand veins of healthy volunteers (Bhagat *et al*, 1995). There was no particular reason to suppose that this would be the case, however, given the pluripotentiality of arachidonic acid alluded to in chapter 1, and the variability of results from animal studies alluded to in chapter 3. These two pieces of evidence imply that vasodilator metabolites of arachidonic acid predominate in the circulation of man, potentially across a wide spectrum of health and disease, and across a wide spectrum of vasoactive therapy. We have no information on whether the response demonstrated is endothelium-dependent or endothelium-independent, but it does not appear to be significantly impaired by heart failure (in patients with presumed endothelial dysfunction, even though we did not demonstrate this independently). It would obviously have been interesting to correlate the effect we observed with more conventional vasodilator responses, such as the response to acetylcholine versus nitroglycerin. Our overriding interest, in examining the effects of arachidonic acid, however, was to find a vascular response which might act as a substrate for the vascular effects of aspirin. As we have repeatedly observed, aspirin does not appear to have any immediate direct vasoactive effect on its own, so elucidation of its vascular effects does appear to necessitate examination of more complicated interactions. Examination of the effect of aspirin on the response to arachidonic acid is the perfect target for this, as any vasoactive effects that aspirin does have must occur via its effect on cyclooxygenase, and therefore on metabolism of arachidonic

acid (Vane *et al*, 1971). The fact that we demonstrated that aspirin did impair vasodilation to arachidonic acid in patients with heart failure treated with an ACE inhibitor does not *prove* that aspirin affects vascular tone in these patients, but it does prove that it *could*, even at a *very* low dose. In our subsequent studies, we have attempted to home in on some of the pathways that might be involved if aspirin does affect vascular function in heart failure. In some of our studies, we have directly assessed the effect of aspirin therapy itself, but in some of them we have only directly assessed the effect of conventional heart failure treatment.

10.2 Chapter 4 Interaction of aspirin and furosemide in heart failure

The studies described in chapter 4 demonstrate that furosemide (a mainstay of the treatment of heart failure) has acute venodilator effects which may be blocked by clinically relevant doses of aspirin (indeed, in this case, they were reversed). In case it could be claimed that the effect observed was not clinically relevant, we compared our results with the effect of sublingual glyceryl trinitrate (the archetypal vasodilator in clinical practice). We found that the vasodilator response demonstrated with furosemide was very similar to that with GTN (with a similar effect on venous capacitance and a similar lack of effect on forearm blood flow, pulse and blood pressure). Furthermore, these results are strikingly similar to our results in chapter 3 (with significant inhibition by both 75mg and 300mg aspirin, and no significant difference between the two, although there was more inhibition by 300mg than 75mg) and serve to provide compelling validation of both sets of results.

10.3 Chapter 5 Effect of bradykinin and substance P in heart failure

The studies described in chapter 5 demonstrate that bradykinin and substance P are both potent vasodilators in patients with heart failure treated with an ACE inhibitor. More importantly, from our point of view, we showed that neither of them is significantly affected by aspirin, whether intra-arterial or oral (at a dose shown in chapter 3 to be *capable* of affecting vascular responses). Both sets of findings are a little controversial. There is some controversy over whether the defect in endothelial dysfunction is specific or generalised. In part, this relates to the difficulty of dissociating stimulated vascular responses from the effect of basal vascular tone. If basal blood flow is reduced (as it usually is in heart failure and other causes of endothelial dysfunction, even on treatment), it is obvious that absolute increases in blood flow cannot be taken as representative, but there is no guarantee that relative increases in blood flow are any more representative. In any case, our findings *suggest* that the responses to bradykinin and substance P are not significantly impaired in patients with heart failure. This suggestion is tentative because we did not have a control group for comparison, and it is comparison with previous work which makes the suggestion. We can be more definite about the fact that aspirin did not affect the response to bradykinin or substance P. Although it is a commonplace of vascular biology that bradykinin is responsible for prostaglandin-dependent, non-angiotensinergic effects of ACE inhibitors, the evidence that the vasodilator effects of bradykinin are prostaglandin-dependent is already inconsistent, as we have pointed out in chapters 3 and 5. Our findings in chapter 5 make it even less likely that the effects of bradykinin (or substance P) are affected by aspirin, even if bradykinin does contribute to the beneficial effects of ACE inhibitors. In other words, bradykinin and

substance P are unlikely to contribute to the interaction between the effects of aspirin and the effects of ACE inhibitors (if indeed such an interaction exists).

10.4 Chapter 6 Effects of bradykinin antagonism in heart failure

In chapter 5 we showed that bradykinin is unlikely to be affected by aspirin. In chapter 6 we looked at the other side of the proposed interaction between aspirin and ACE inhibitors. It is well-established that ACE inhibitors potentiate the effects of exogenous bradykinin. Despite this, there is little evidence that potentiation of endogenous bradykinin actually contributes significantly to the beneficial effects of ACE inhibitors. The studies described in chapter 6 demonstrated that an antagonist of endogenous bradykinin had no discernible effect on vascular tone in patients with heart failure. This was irrespective of treatment with an ACE inhibitor (which might have been expected to potentiate endogenous bradykinin) or an AT1 antagonist (which might not, at least not directly). We should be careful not to over-interpret these findings, especially in the light of a recent publication which showed that an antagonist of endogenous bradykinin attenuated the haemodynamic effects of an ACE inhibitor given to patients with hypertension (Gainer *et al*, 1998). We should be careful not to over-interpret those findings either, as there are many differences between the two studies which certainly raise as many questions as they answer. In any case, our findings in chapter 6 (together with our findings in chapter 5) imply that the suggestion that bradykinin is an intermediary between the effects of ACE inhibitors and aspirin should be treated with extreme caution.

10.5 Chapter 7 Effect of angiotensin-(1-7) in heart failure

Having disposed of bradykinin and substance P as potential mediators of an interaction between aspirin and ACE inhibitors, we turned our attention to the greater complexity of the renin-angiotensin-system itself. As we observed in chapter 1, there are a large number of angiotensins, which had previously been regarded as mere degradation products. More recently, it has been realised that many of these are detectable in the circulation, that many of them are actually potentiated by treatment with ACE inhibitors, and that some of them are certainly vasoactive. Despite this, there is almost no work on humans, and none in heart failure. Most of the recent interest has focussed on the effects of angiotensin-(1-7). In particular, animal work has included complex interactions of angiotensin-(1-7), ACE inhibitors, bradykinin and prostaglandin synthase inhibitors (indomethacin, rather than aspirin). Despite this, we found that we could not sustain a significant vasoactive effect of angiotensin-(1-7) in the vasculature of patients with heart failure treated with an ACE inhibitor. Although it is possible that we would have obtained a different result in patients not treated with an ACE inhibitor, we were able to point out that our findings were consistent with the very small body of work in humans, both normal and hypertensive, treated with an ACE inhibitor and not. It certainly seems unlikely that angiotensin-(1-7) contributes to any interaction between aspirin and ACE inhibitors.

10.6 Chapter 8 Plasma fibrinolytic variables in heart failure

As we pointed out in chapter 8, there is extensive evidence that conventional heart failure treatment in the form of ACE inhibitors affects plasma fibrinolytic parameters. There is also some evidence that more modern heart failure treatment in

the form of AT1 antagonists affects plasma fibrinolytic parameters differently. If this *is* the case, it must presumably reflect a non-angiotensinergic effect, or at least a non-angiotensin-II-ergic effect. If that is the case, it might be a prostaglandin-dependent effect, which might interact with the effects of aspirin. We took great care to study our patients off aspirin therapy (care which previous studies appear not to have taken), and found no evidence of any difference between ACE inhibitors and AT1 antagonists in this regard. There seemed little gain, therefore, from examining the additional effect of aspirin therapy, if any, on our treatment. It might still be interesting to do so. It is obvious that aspirin, through its antiplatelet effects, has complex interactions with the circulating fibrinolytic system, and indeed aspirin has been shown to enhance plasma fibrinolytic variables (Moroz, 1977). Despite this, we are not aware of any reports of the effect of aspirin on fibrinolysis being mediated via effects on endothelium.

10.7 Chapter 9 Plasma nitrate/nitrite concentrations in heart failure

Although we did not have the opportunity to examine the interaction of any of our treatment with conventional measures of endothelial function (for example, the response to acetylcholine versus nitroglycerin), we were able to examine the effect on nitrate/nitrite levels. This is obviously an indirect measure of endothelial function, but has been at least partially validated in this regard. We found that there was no significant difference between ACE inhibitor and AT1 antagonist treatment. To date, there has been very little comparison of endothelial function between ACE inhibitors and AT1 antagonists, especially in heart failure, so this is a useful piece of information, despite its limitations. In particular, we have no information on whether

this was because the two treatments were equally effective, or equally ineffective. If there really is no difference in endothelial function between patients treated with ACE inhibitors and patients treated with AT1 antagonists, it makes it less likely that the improvement in endothelial function with ACE inhibitors (if any) is due to potentiation of bradykinin, unless AT1 antagonists are potentiating bradykinin in some way as well. Of course, our findings in chapter 5 cast doubt upon the potentiation of bradykinin itself, by either ACE inhibitors or AT1 antagonists. We were not able to examine the effect of aspirin on this aspect of endothelial dysfunction. It would have been interesting to do so, given the conflicting reports in this regard. Once again, however, the lack of any difference between ACE inhibitors and AT1 antagonists whilst off aspirin therapy weakened our resolve to look for a difference between them whilst on aspirin therapy.

10.8 Further studies

We have not completely characterised the effects of arachidonic acid, nor have we completely characterised the effects of heart failure on the response to arachidonic acid. Apart from correlating the effects of arachidonic acid with more conventional measures of endothelial dysfunction, it would be interesting to test arachidonic acid in a wider patient group, including an appropriate control group (difficult as it can be to define an appropriate control group for heart failure patients). It would also be interesting to test the effect of lower doses or different formulations of aspirin (such as modified-release or dermal aspirin). Similarly, we have not characterised the effects of bradykinin and substance P as completely as we might, with an appropriate control group. Our findings with bradykinin-antagonism and enalapril and losartan

are amongst our most interesting findings, not least because they are at odds with our expectations and previous findings. Our hypothesis that the difference between our findings and previous findings is due to timing deserves to be tested, with simultaneous examination of acute and chronic effects under more similar conditions (this would also allow re-examination of plasma fibrinolytic parameters to resolve a similar inconsistency). It is also possible that the lack of effect of bradykinin-antagonism is down to a lack of effect at rest. We would like to examine the interaction, if any, between bradykinin antagonism and flow-dependent dilatation and any influence of enalapril and losartan. It would certainly be interesting to test the effect of angiotensin-(1-7) in a different patient group, as we found no effect in patients with heart failure treated with an ACE inhibitor. We would also like to examine the effect, if any, of A-779 which is a recently available antagonist of angiotensin-(1-7).

10.9 Summary

Our main interest is in the vascular effects of aspirin and ACE inhibitors (and AT1 antagonists) in patients with heart failure. Chapter 3 showed that arachidonic acid and its metabolites may be important in the blood vessels of patients with heart failure and that doses of aspirin in common use for cardioprotection have the potential to interfere with this system. Chapter 4 showed that the same doses of aspirin had similar effects on the venodilator response to furosemide in a completely different vascular bed. Chapter 5 showed that despite this, there was no evidence that aspirin had any effect on the potent vasodilators, bradykinin or substance P. Chapter 6 showed that there was little evidence that ACE inhibitors (or indeed AT1 antagonists)

caused significant potentiation of endogenous bradykinin anyway. Chapter 7 showed that angiotensin-(1-7) was also unlikely to contribute to the effects of ACE inhibitors (or indeed AT1 antagonists) as it seems to be inactive after all. Chapter 8 showed that there was no significant difference between ACE inhibitors and AT1 antagonists in terms of their effects on plasma fibrinolytic variables. Chapter 9 showed that there was no significant difference between ACE inhibitors and AT1 antagonists in terms of their effect on overall endothelial function in terms of plasma nitrate/nitrite levels (which further militates against a role for bradykinin and/or prostaglandins). Taken together these results suggest that whilst it is relatively easy to demonstrate that aspirin could have detrimental effects in the vasculature of patients with heart failure, it is very much more difficult to demonstrate that it is actually doing so. Similarly, whilst it is relatively easy to conceive of differences between ACE inhibitors and AT1 antagonists, it is very much more difficult to demonstrate that there actually are any. Our findings confirm our view that whilst the vascular effects of aspirin are almost certainly quite simple, the vascular effects of ACE inhibitors and AT1 antagonists are really quite complicated, yet much more difficult to dissociate from one another than we had at first anticipated.

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